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Characterizing the adhesive properties of cytokine receptor IL17Ra during brain development

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ABSTRACT

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder that is mainly characterized by three different deficits – social behavior, communication, and characteristic repetitive behaviors. Many human studies revealed the significant effect of environmentally-induced maternal immune activation (MIA) during pregnancy in the pathogenesis of ASD, especially in genetically vulnerable individuals. This observation has been modeled in MIA rodents to better understand the neural basis of MIA-induced ASD deficits. Although many studies have hinted at the importance of the T helper 17 (Th17) cell pathway in the pathogenesis of ASD, the exact immune cell populations responsible for inducing ASD-like phenotypes in MIA animal models have not been identified. A recent study revealed that interleukin-17a (IL-17a), the effector cytokine of Th17 cells, is a critical regulator of neurodevelopment, thus suggesting the importance of its receptor IL17Ra in causing ASD-like phenotypes in the MIA models as well.

In this study, we aimed to characterize the role of the cytokine receptor IL17Ra in various neurodevelopmental processes. We had hypothesized that the protein potentially acts as a cell adhesion molecule to critically regulate the cellular phenotypes during cell adhesion, neurite outgrowth, and synapse formation. Contrary to our hypothesis, we demonstrated that IL17Ra inhibits, rather than stimulates, cell adhesion, as cell adhesion levels of IL17Ra-overexpressing non-neuronal cells were lower than those of control cells. IL17Ra expression in wild type primary cortical neurons also suppressed neurite outgrowth and synapse puncta formation, further supporting the inhibitory role of the protein. Possible mechanisms by which IL17Ra is involved in neurodevelopment and the implications will be discussed. Further elucidation of the role of IL17Ra could lead to more definitive therapeutic and preventative tools for ASD, as targeting of Th17 cells in conjunction with regulating IL17Ra expression in susceptible pregnant mothers may allow us to reduce the likelihood of bearing children with inflammation-induced ASD phenotypes.

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CHAPTER 1: INTRODUCTION

Autism spectrum disorder (ASD) and its etiology

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder that is clinically characterized by social impairments, communication delay, and stereotypical repetitive behaviors (*1*). This disorder affects approximately 1 percent of the world population and has been reported in all socioeconomic and ethnic groups (*2, 3*). In the United States alone, ASD affects more than 3.5 million lives, and ASD services for U.S. citizens are estimated to cost $236-262 billion annually (*4*). It is critical to further elucidate the causes, potential therapeutic treatments, and preventative methods of the disorder to better treat the large population of individuals affected with ASD.

The etiology of ASD is heterogeneous – that is, both genetic and environmental factors contribute to its pathogenesis (*5*). For example, numerous studies have reported strong genetic contributions to the cause of ASD. First, approximately 10% of individuals affected with ASD also display symptoms of other genetic, neurologic, or metabolic disorders, such as fragile X syndrome and tuberous sclerosis, revealing genetic associations between ASD and various disorders (*6, 7*). In addition, the risk of ASD in newborns is 25 times greater than that in the general population if their siblings are affected with ASD, which strongly marks genetics as one of the root causes of ASD (*8*). Furthermore, two different twin studies revealed that identical twins have 60-90% concordance rate of ASD, compared to 0-24% in fraternal twins (*9, 10*). Finally, although more than 20 different chromosomes have autism loci, these only account for the subset of ASD population with other genetic syndromes, and not those with only ASD, implying ASD is multifactorial (*6*).

Alarmingly, the occurrence of ASD in the world has been sharply increasing over the past two decades. From 1992 to 2007, the estimated rate of incidence of ASD in the U.S. increased from 1 in 500 children to 1 in 110 children. In 2015, ASD was recently estimated to affect 1 in 45 children (*11, 12*). Other childhood immunological disorders, such as asthma, life-threatening food allergies and autoimmune disorders, have reached epidemic levels in parallel to the increase in ASD (*13*). It is unlikely that the significant upward trends in these diseases can be attributed solely to genetics, as two decades is not nearly enough time for genetic changes to have a severe impact on the prevalence of various disorders at the population scale (*14*). Although the apparent increase in prevalence of these diseases may, in part, be attributed to heightened awareness, increase in knowledge, and the broadening of diagnostic criteria of the disorders, it has been hypothesized that environmental factors may have much greater roles on the pathogenesis of ASD than previously expected (*11*).

Consistent with this idea, many recent studies attribute the cause of the rapid increase in individuals with ASD today to the rise in environmental stressors during critical periods of neurodevelopment, especially in genetically vulnerable individuals (*15*). More specifically, immune disturbance due to various environmental factors like psychological stressors, chemical exposures, and pharmaceuticals are candidate risk factors of ASD, as these factors may ultimately alter the maternal immune responses during prenatal development and detrimentally affect neurodevelopment (*11*).

Maternal immune activation (MIA)

Maternal immune activation (MIA) refers to the activation of the immune system in the mothers during gestation due to immune-related disruptions such as bacterial and viral infections. Many epidemiological studies emphasize the role of MIA in the pathogenesis of ASD. For

example, the correlation between ASD and maternal viral infection during gestation was first noted when the number of individuals affected with ASD escalated from 0.05% to 8-13% after the 1964 rubella pandemic (*16*). More recently, an epidemiological study of over 10,000 records in the Danish Medical Birth Register revealed a strong correlation between prenatal infections in the mothers during their first and second trimesters of pregnancy, and a nearly threefold increase in frequency of ASD in their children born from those pregnancies (*17*). The results from these studies suggest the high likelihood of immune molecules playing critical roles in the pathogenesis of ASD.

Although the specific mechanisms by which infections cause latent pathology in the central nervous system of the offspring are still unknown, previous research has reported that MIA and the maternal pro-inflammatory cytokine, rather than direct infection of the fetus, interfere with fetal neurodevelopment to induce ASD phenotypes (*18*). Accordingly, a study showed direct alterations in the concentrations of various maternal cytokines in relation to diagnosis of ASD in the children later in life. More specifically, the results showed that mothers who had borne children with ASD had increased mid-gestational levels of cytokines interleukin (IL)-4, IL-5, and interferon-gamma (IFN-gamma), compared to mothers who had not (*19*). An epidemiological study of the Danish Historic Birth Cohort also revealed increased concentrations of IL-4 and IL-5 in the amniotic fluids of the mothers that gave birth to children affected with ASD after, but not before, 1993. Although this contrast may be due to improvements in diagnostic criteria and is difficult to explain, the overall conclusions still imply that cytokines are critically involved in the pathogenesis of ASD (*20*). Together, these studies suggest that MIA during prenatal development can have deleterious effects on proper brain development, and ultimately on cognitive skills in the offspring. More specifically, the strong association between

MIA in pregnant mothers and ASD in the children born from these pregnancies implies that the changes in concentrations of cytokines that facilitate the maternal immune responses may be detrimental to neurodevelopment in the subsequent generation (*18, 21*).

Cross-talk between the mother and embryo

The exact mechanism by which the increased cytokine levels in the mothers after immune challenges affect brain development and behavior is still not well understood. However, we can hypothesize how certain immune molecules may cross the barrier to affect neurodevelopment, since the development of the maternal fetal exchange system, or utero-placental circulation system, is well characterized. Upon implantation of the fertilized ovum, establishing proper connections to the mother is a critical step for the development of the embryo (*22*). At this stage, however, the embryo receives its necessary nutrients via simple diffusion. As the pregnancy proceeds, the utero-placental circulation system develops to control and regulate gas and nutrient exchange between the mother and the embryo. Placenta, an autonomous and transient organ, is formed during pregnancy and is responsible for physically connecting the embryo to its mother and facilitating maternal-embryonic exchange (*23*). The development of placental structures results in physical and functional connections, which facilitate hormonal regulations that direct the changes in the uterus and establish a hybrid vasculature system by which maternal blood transports material such as cytokines, nutrients, and gases to the embryo (*22, 23*).

During the first trimester of the pregnancy, the endothelium of the placental villus differentiates and forms a placental barrier that regulates maternal-embryonic exchange. By the end of first trimester, the placental barrier consists of four layers that the gases, nutrients, and waste of the maternal and fetal blood must diffuse through (*24*). The embryo grows rapidly, and

during the second trimester, the endothelium of the villus adapts to this growth by dividing further. As a result, the endothelium of the villus becomes very thin, and the intervillus space between the maternal and fetal blood decreases. It is important to note that the maternal and fetal blood rarely come into contact, (though some cases of direct exchange have been observed), and molecules that are transferred to the fetus, and vice versa, are very carefully modulated (*25*). However, by this point of the second trimester, various molecules can relatively easily cross the placental barrier because the placental precursors are mono-layered and thin, compared to later in placental development (third trimester), when the barrier differentiates further and becomes more selective (*26*). It is believed that it is during the second trimester that the embryo is most susceptible to MIA, and this could possibly explain the high correlation of viral/bacterial infections during the second trimester of pregnancy in the mother with ASD in the offspring.

Maternal immune activation (MIA) rodent models

Since the establishment of maternal infection as a candidate risk factor for ASD, MIA has been modeled in rodents to better understand the neural basis of MIA-induced ASD deficits (*27*). Several rodent models of MIA have been created by maternal injections of either polyinosine:cytosine [poly(I:C)] or lipopolysaccharide (LPS) to induce the adverse transformations during brain development (*27*). Poly(I:C) is a synthetic double-stranded RNA that mimics the acute phase response of viral infections in the absence of the pathogen. Upon intraperitoneal injection, poly(I:C) targets maternal toll-like receptor (TLR) 3 of dendritic cells, a type of cell in the immune system, and activates downstream pathways to mimic the maternal immune response (*28*). In the second MIA rodent model, LPS, which is introduced via intrauterine injection, targets TLR4 of dendritic cells to induce similar ASD-like behavioral abnormalities seen in offspring of poly(I:C)-injected mothers (*27, 28*). These MIA models have

been widely adopted and many studies have utilized these MIA animal models to reproduce the core symptoms of ASD (*29*).

MIA models help elucidate the roles of certain cytokines involved during maternal immune responses, as well as the implications of their elevated levels in causing ASD-like phenotypes (*28*). For example, a recent study of a pro-inflammatory cytokine IL-6 showed that IL-6 alone is necessary and sufficient to induce ASD-like neuropathological and behavioral changes in the MIA offspring of dams. More specifically, MIA-induced ASD-like phenotypes were eliminated when IL-6 was blocked in the mothers, and IL-6 exposure during gestation in the absence of poly(I:C) MIA induction was sufficient to cause ASD-like phenotypes in the offspring. This study strongly suggests the central role of MIA in causing long-term, ASD-like neuropathological and behavioral changes in the offspring (*18*).

T helper (Th) 17 / IL-17 pathway

Although the necessity of maternal cytokine IL-6 in the induction of ASD-like phenotypes is well recognized, the exact immune cell populations responsible for the induction of ASD-like cortical and behavioral phenotypes in the fetus have not been identified (*18*). Several studies recognize the downstream mediators of IL-6, Th17 cells and its corresponding cytokine IL-17, as the candidate immune cell population and molecule responsible for the pathogenesis of ASD in the offspring. First of all, Th17 cells have been known to play a role in the induction of inflammatory and autoimmune diseases such as asthma, rheumatoid arthritis, psoriasis, inflammatory bowel disease and multiple sclerosis, suggesting the central role of the Th17/ IL-17 pathway in causing various immune-related diseases (*30*). A recent study revealed that the concentration of IL-17a, which is secreted by Th17 cells, is increased in the sera of a group of ASD children, and a genome-wide copy number variant (CNV) analysis of ASD

patients revealed that *il17a* is enriched and dysregulated (*31, 32*). These data all suggest that the Th17 cells and their cytokines may be the candidate immune cell population and molecules that directly affect neurodevelopment of the offspring and therefore induce ASD. Further characterization of the Th17/ IL-17 signaling pathway would allow better understanding of the molecular basis of how ASD-like behavioral deficits are induced via MIA.

Most recently, the Choi lab at the Massachusetts Institute of Technology has identified the cellular source that is responsible for causing ASD-like cortical and behavioral phenotypes in the offspring of the MIA model. More specifically, we showed that IL-17 is necessary and sufficient to induce ASD-like phenotypes (*33*). We found that IL-17a levels in maternal sera are elevated upon poly(I:C) injection, and that pre-treatment of mothers with antibodies blocking IL-17a before injection restores elevated cytokine levels (Fig. 1A). Importantly, we also found elevated IL-17Ra mRNA levels in fetal brains at E14.5 in fetus of mothers injected with poly(I:C), suggesting that maternal IL-17 directly enters and amplifies the signaling in the fetal brain (Fig. 1B). Furthermore, abnormal cortical development was observed in affected fetus at E14.5, as shown by decreased levels of SATB2 expression levels, a marker of postmitotic neurons in superficial cortical layers. This abnormality was rescued by inhibition of IL-17a signaling via pre-treatment with antibodies (Fig. 1C). Finally, abnormal cortical morphology was observed in afftected fetus at E18.5, and was rescued when the mothers were pre-treated with antibodies blocking IL-17a (Fig. 1D). These results together not only suggest that the Th17 cell/IL-17 pathway is involved in inducing ASD-like phenotypes, but also show that IL-17 can directly affect neurodevelopment and induce adverse cortical transformations in the fetal brain to cause ASD-like phenotypes.

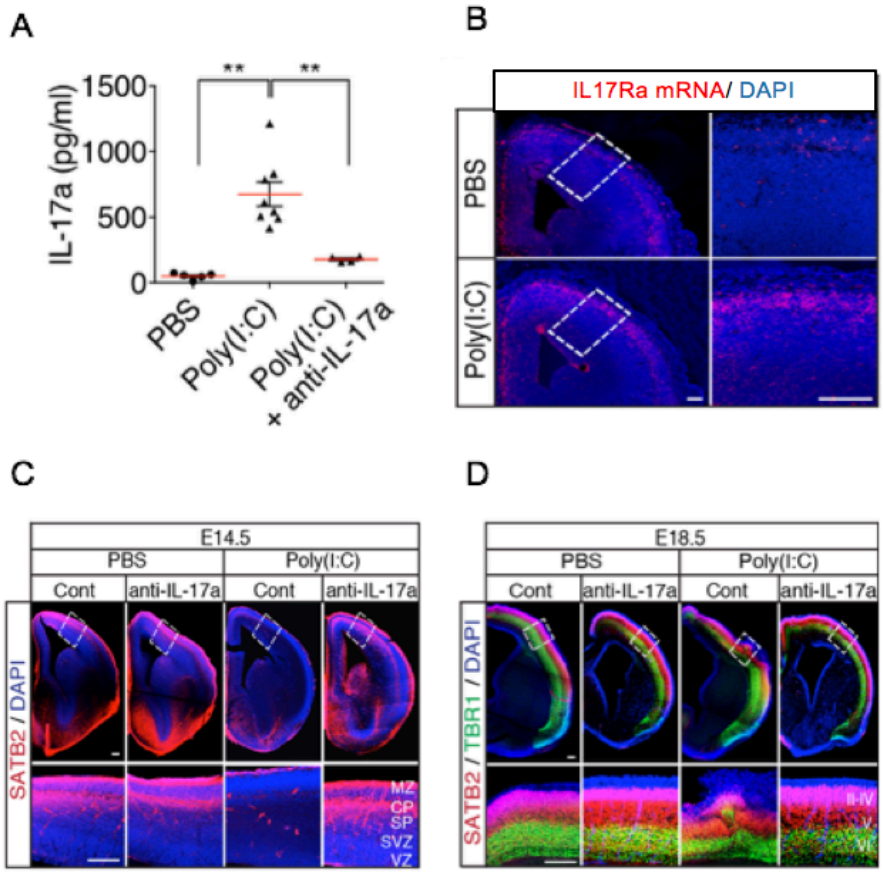


Figure 1: MIA-induced mothers express elevated IL-17a levels, leading to elevated IL-17Ra mRNA levels and abnormal cortical development in the offspring. (A) Serum concentrations of maternal IL-17a at E14.5 in PBS (control) or poly(I:C)-injected mothers, pretreated with isotype control or IL-17a blocking antibodies. (B) *In situ* hybridization with an IL-17Ra RNA probe in E14.5 fetal brains derived from PBS- or poly(I:C)- injected mothers. (C) Immunofluorescence staining of SATB2 (a marker of postmitotic neurons in superficial cortical layers) in E14.5 fetal brain, derived from PBS- or poly(I:C)-injected mothers, pretreated with isotype control (Cont) or IL-17a blocking antibodies (anti-IL-17a). Scale bar represents 100m. (MZ: marginal zone, CP: cortical plate, SP: subplate, SVZ: subventricular zone, VZ: ventricular zone) (D) SATB2 and TBR1 (a marker restricted to deeper cortical layers) staining in E18.5 fetal brains from animals treated as in (C). Adapted from Choi et al., 2016.

According to Choi et al., inflammation in the mother results in upregulation of pro-inflammatory cytokines, such as IL-6, which target and induce differentiation of Th17 cells in the mother's circulating blood. Subsequently, IL-17 cytokines are expressed, which migrate across the placenta via maternal endometrial and umbilical vessels and induce IL-17Ra expression, as well as abnormal cortical phenotype in the fetal brain. This dysregulation of cytokine concentrations may explain the behavioral abnormalities observed in the MIA-induced ASD-like offspring (Fig. 2) (*33*). The specific role of IL17Ra and its molecular consequences, however, are still unknown.

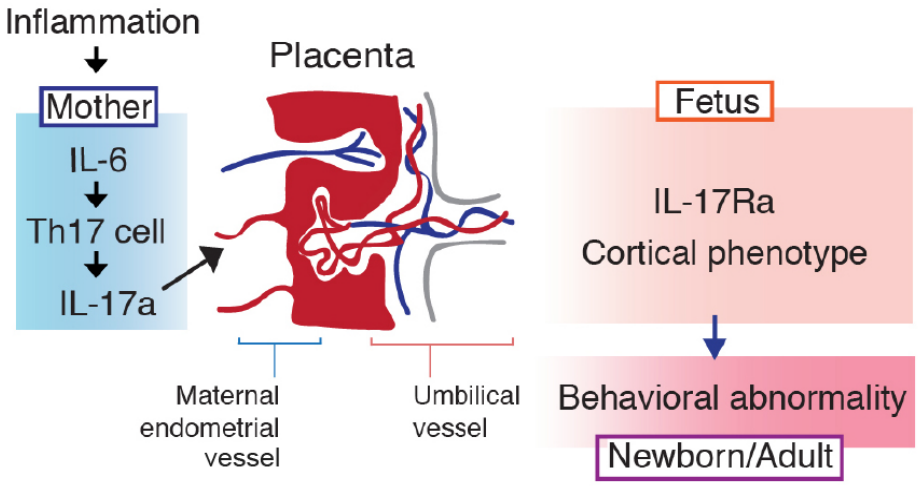


Figure 2: The proposed mechanism by which the maternal Th17/IL-17 pathway induces MIA-dependent behavioral and cortical abnormalities in the offspring. Based on mouse experiments, maternal cytokine IL-6 levels increase upon inflammation. IL-6 targets and initiates differentiation of Th17 cells, which produce IL-17a. IL-17a travels through the selective placental barrier via maternal endometrial and umbilical vessels, and Il17Ra expression in the fetal brain is increased. The increase of IL17ra results in amplified IL-17 signaling in the fetal brain, and leads to ASD-like cortical and behavioral phenotypes in the offspring. Adapted from Choi et al., 2016.

As previously stated, recent studies show that both concentrations of maternal IL-6 and its downstream mediator IL-17 are elevated, and that either cytokine is necessary and sufficient to cause ASD-like phenotypes in the offspring (*18, 33*). These results raise the question of which cytokine directly affects the offspring and is responsible for inducing ASD-like phenotypes. In order to determine the specific immune molecule that is at play, we directly injected either IL-6 or IL-17 into the fetus and observed its effects on neurodevelopment. Only the IL-17 injected fetal group displayed MIA-induced ASD-like phenotypes, indicating that although IL-6 and IL-17 both mediate the maternal immune response, the placenta-blood barrier is selective and only IL-17 was able to penetrate into the fetal brain to directly affect neurodevelopment (unpublished data). Further studies of the IL-17 pathway are needed to elucidate the molecular mechanisms by which IL-17 signaling adversely affects neurodevelopment in the fetus.

MIA, neurodevelopment, and ASD

Abnormalities of cortical phenotype in the MIA offspring suggest that MIA disrupts normal cortical development during the critical period of brain development. The period of corticogenesis in mice (E11-19) overlaps with the time period during which the fetus is most susceptible to the actions of MIA (*33, 34*). Cortical development encompasses the entire process of proliferation, migration, differentiation, synaptogenesis, myelination, gliogenesis, and apoptosis of neural cells, indicating multiple time points at which proper neurodevelopment can be disrupted. Throughout the entire process of cortical development, cytokines play critical roles in regulating the intercellular interactions during development and functioning of the cortical layers (*35*).

In neocortical development, proliferation first begins by symmetrical division of the neuroepithelial cells in the ventricular zone. Then, these precursor cells become radial glial cells,

which are multipotent progenitors of neural cells, and either serve as expanding cells with processes that the neural cells migrate along, or become neurogenic cells (*35, 36*). Newborn neurogenic cells migrate along the radial glial processes, and arrive in their correct layers in an inside-out manner, with earlier born cells populating the deeper layers of the cortex, and later born cells populating the superficial layers of the cortex. Upon arrival, the cells differentiate and form complex neural circuitry that underlies the function of the cortices. Furthermore, microglia, or immune surveillance cells with hematopoietic origin, play critical roles during development by clearing apoptotic debris and modulating vascularization, survival and synapse formation and function, in part through their response to, and secretion of, various cytokines (*37*). A small subclass of cytokines regulates these corticogenic processes. For example, IL-1 is known to regulate proliferation of progenitor cells, while IL-6 and IL-10 are thought to regulate oligodendrocyte and astrocyte maintenance to ultimately facilitate neuronal growth (*37*). These processes overlap with periods in which the fetus is most vulnerable, and the dysregulation of cytokine concentrations, and consequently disruption of proliferation, migration, or differentiation, will most likely result in abnormal cortical development (*35, 37*).

Based on previous studies, as well as our preliminary data, here we propose the possible mechanism by which the IL-17a pathway disrupts proper migration, axon pathfinding and synapse formation during cortical development to induce abnormal cortical and behavioral phenotypes. More specifically, we hypothesize that IL-17Ra acts as a cell adhesion molecule during cortical development due to several reasons. First, IL17Ra's topology is homologous to other well-characterized cell adhesion molecules, many of which have extracellular, transmembrane, and intracellular domains (*38*). Furthermore, the extracellular domain of the protein has a fibronectin type III (FNIII) domain, which is well known to be involved in various

processes during neurodevelopment, such as cell adhesion, migration, and morphogenesis (Fig. 3) (*39*). Additionally, it is worthwhile to note that many well studied cell adhesion molecules like neural cell adhesion molecule (NCAM), synaptic cell adhesion-like molecule (SALM), and roundabout (Robo) also express FNIII domains. Finally, it is important to note that different sets of cell adhesion molecules regulate different neurodevelopmental processes, and thus it is important to conduct many assays to determine the specific role of the protein (*40*). For example, another cytokine of the interleukin family, IL-1, is known have adhesive properties and play active roles during brain development. Its receptor, IL-1RAcP, was found to act directly as a cell adhesion molecule that organizes neuronal synaptogenesis. Interestingly, the intracellular domain of IL17Ra has a SEFIR domain, which is similar in length and secondary structure as the toll/interleukin-1 receptor (TIR) domain of the IL-1RacP (Fig. 3) (*39, 41*). Based on previous studies and the homology between IL17Ra and other well-characterized cell adhesion molecules, we hypothesize that IL17Ra acts as a cell adhesion molecule during cortical development.

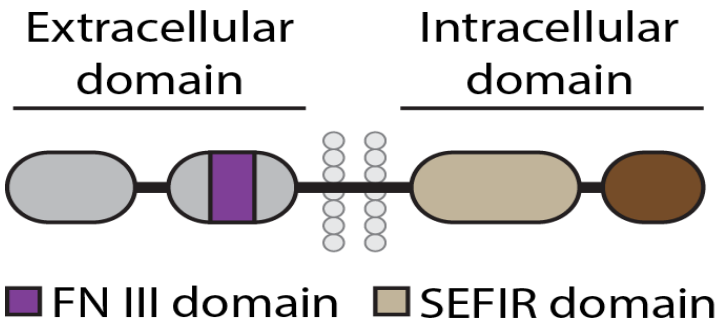


Figure 3: Topology of IL17Ra. IL17Ra has extracellular, transmembrane, and intracellular domains. The extracellular domain of IL17Ra has a fibronectin type III (FNIII; purple) domain, and the intracellular domain has a SEFIR domain (light brown).

Thesis aims

Here, we aim to test whether IL17Ra, a cognate receptor of IL-17, is an adhesion molecule that facilitates various neurodevelopmental processes such as neurite outgrowth, axon pathfinding, and synapse formation, and if so, we will attempt to determine which of these processes the protein facilitates. If IL17Ra has an indirect role in cell adhesion, activation of IL17Ra will lead to activation of downstream pathways that will stimulate the action of other endogenous cell adhesion molecules (Fig. 4A). If IL17Ra has a direct role in cell adhesion, activation of IL17Ra itself will stimulate cell adhesion via homophilic binding to itself or via heterophilic binding to other endogenous cell adhesion molecules (Fig. 4B). Although further experiments are required to determine the exact mechanisms, if IL17Ra acts as a cell adhesion molecule, either case would lead to increased facilitation of cell adhesion, and therefore increased cell aggregation and synapse formation.

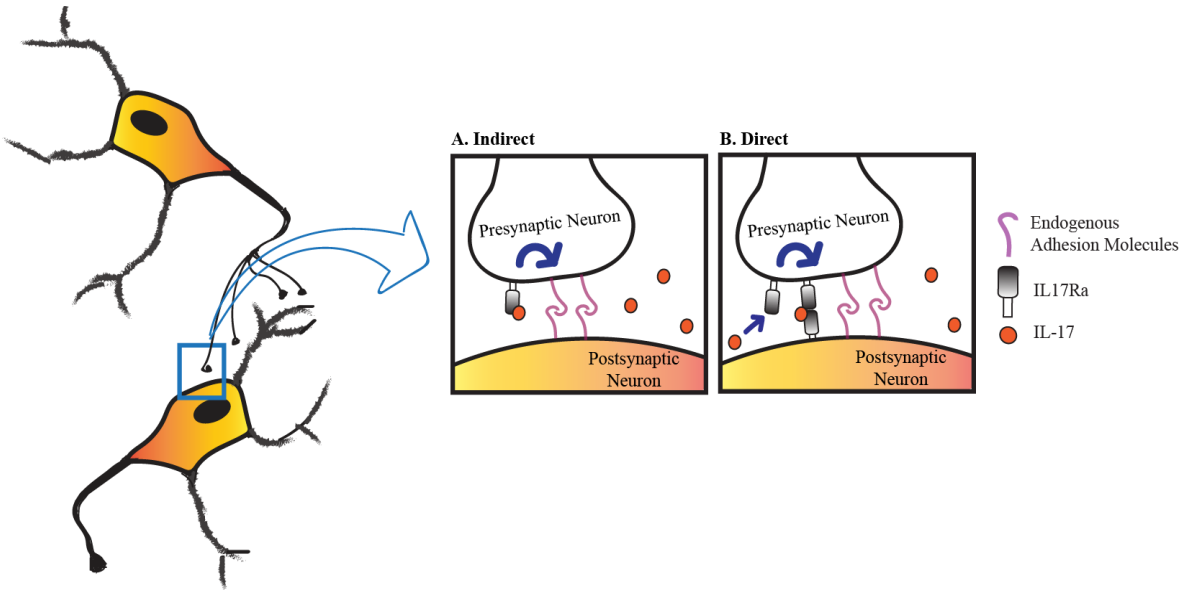


Figure 4: The potential role of IL17Ra as a cell adhesion molecule. The diagram illustrates the indirect (A) and direct (B) mechanism by which IL17Ra may act as a cell adhesion molecule to facilitate cell adhesion.

In order to test this hypothesis, we will use four different assays – cell aggregation assay, neurite outgrowth assay, synapse puncta assay, and artificial synapse formation assay.

1. *Cell Aggregation Assay*

The cell aggregation assay aims to determine whether IL17Ra can facilitate stable cell-to-cell interactions by transfecting IL17Ra onto human embryonic kidney 293 (HEK293) cells. In HEK293 cells with endogenous levels of IL17Ra (control group), some level of aggregate will form due to endogenous adhesion molecules and IL17Ra (Fig. 5A). However, if IL17Ra is a cell adhesion molecule, HEK293 cells overexpressing IL17Ra will form larger aggregates than cells that do not (Fig. 5B). On the same note, the presence of IL-17 will induce even greater cell adhesion in IL17Ra overexpressing cells, and therefore will result in even larger aggregates (Fig. 5C).

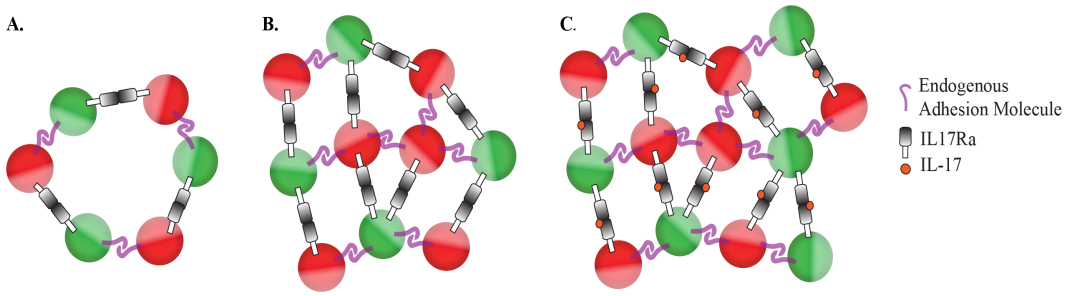


Figure 5: Hypothesized role of IL17Ra in the cell aggregation assay. (A) IL17Ra transfected HEK293 cells, in addition to endogenous adhesion molecules will form larger aggregates than (B) mock vector transfected HEK293 cells with endogenous adhesion molecules and IL17Ra only. (C) The addition of IL-17, the ligand of IL17Ra, will facilitate IL17Ra adhesion and induce greater cell adhesion.

2. *Neurite Outgrowth Assay using primary cultured neurons*

One primary function of neuronal cell adhesion molecules is to regulate axon growth (*42*). If IL17Ra were a potential cell adhesion molecule, one role of the protein may be to aid neurite outgrowth. Therefore, upon loss of function of IL17Ra, we would expect to see impaired neurite outgrowth in primary cortical neurons. Although *in vitro* assays clearly have advantages, such as having greater control over the environmental conditions and the ability to observe homogeneous populations of cells that undergo uniform and synchronous changes, it is important to note that the inherent complexity of *in vivo* systems is lost. Nevertheless, primary neuronal cultures are a powerful tool for analyzing molecular mechanisms by which neuronal development proceeds, and these cultures are commonly used to study various aspects of neuronal development. It is worthwhile to note that many studies have revealed that primary cortical neurons are good representations of *in vivo* neurons in various aspects of neuronal morphology, such as having long processes, maturation, aging, decreased proliferation rates, and death (*43*). Here, we will utilize *in vitro* systems of primary cortical neuron cultures to genetically manipulate and characterize the role of IL17Ra in cortical neuron development.

CRISPR/Cas9 is a genome editing technique that allows easy and specific control of gene expression. We will knockdown endogenous IL17Ra expression in primary neuron culture from the cortex of wildtype fetus using the CRISPR/Cas9 technique, to analyze whether IL17RA loss-of-function affects morphogenesis of primary cultured neurons. We hypothesize that in the LacZ KD control neurons with endogenous IL17Ra expression level, IL17Ra will act as a cell adhesion molecule to aid the process of neurite outgrowth (Fig. 6A). On the other hand, in the IL17Ra KD neurons with IL17Ra loss-of-function, we hypothesize that neurite outgrowth will be impaired (Fig. 6B).

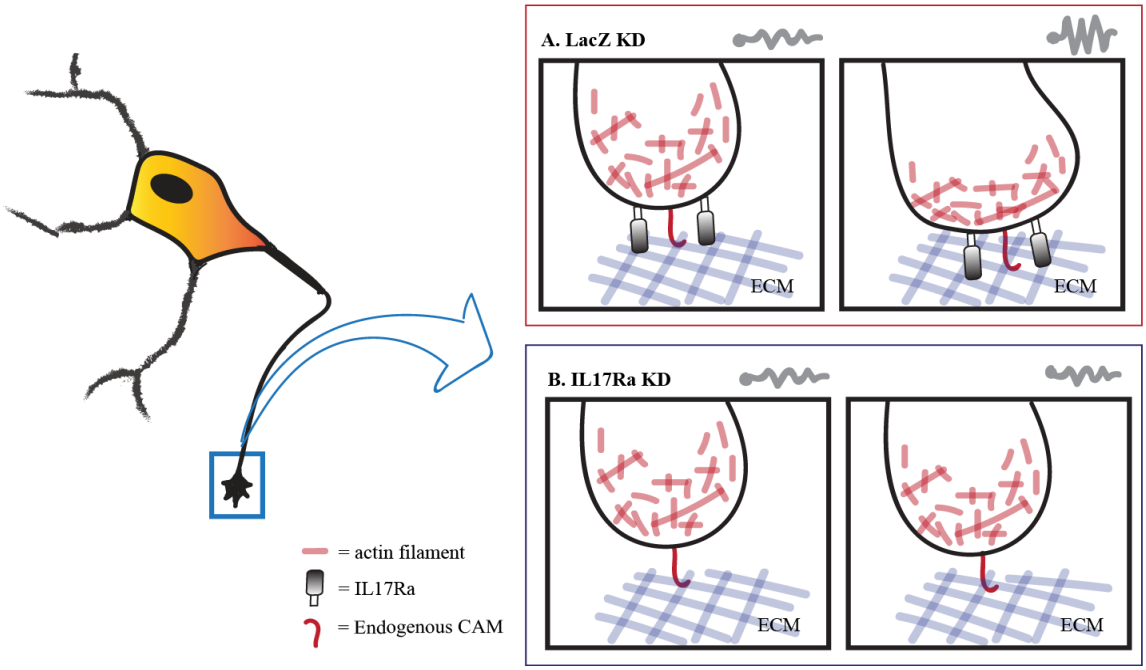


Figure 6: Hypothesized role of IL17Ra in the neurite outgrowth assay. (A) In neurons with LacZ KD, or endogenous IL17Ra expression, IL17Ra will aid in neurite outgrowth by adhering to the proteins in the extracellular matrix (ECM). (B) In neurons with IL17Ra KD, IL17Ra loss-of-function will result in impaired neurite outgrowth.

3. *Primary Cultured Neuron Synapse Puncta Assay*

We will use the CRISPR/Cas9 genome editing technique to also examine the effects of IL17Ra loss-of-function on synaptogenesis of primary cortical neurons. We hyothesize that if IL17Ra acts as a cell adhesion molecule, the protein will aid the process of synapse puncta formation, and that the IL17Ra KD neurons will, therefore, show impaired synapse formation upon IL17Ra loss-of-function.

4. *Primary Cultured Neuron Artificial Synapse Formation Assay*

The artificial synapse formation assay tests whether IL17Ra overexpressing non-neuronal cells can form stable, synapse-like interactions with endogenous IL17Ra and cell adhesion molecules of primary cortical neurons. We will also repeat this procedure using primary cortical neurons from MIA offspring to assess the effects of IL17Ra overexpression in MIA neurons. If IL17Ra aids in synapse formation by acting as a synaptic cell adhesion molecule, we expect to observe increased levels of synapse formation between wild type neurons and IL17Ra overexpressing non-neuronal cells (Fig. 7A) compared to levels in control situations (Fig. 7B). In addition, in MIA neurons that are known to overexpress IL17Ra, we expect to observe further increase in synapse formation compared to that observed in wild type neurons (Fig. 7C).

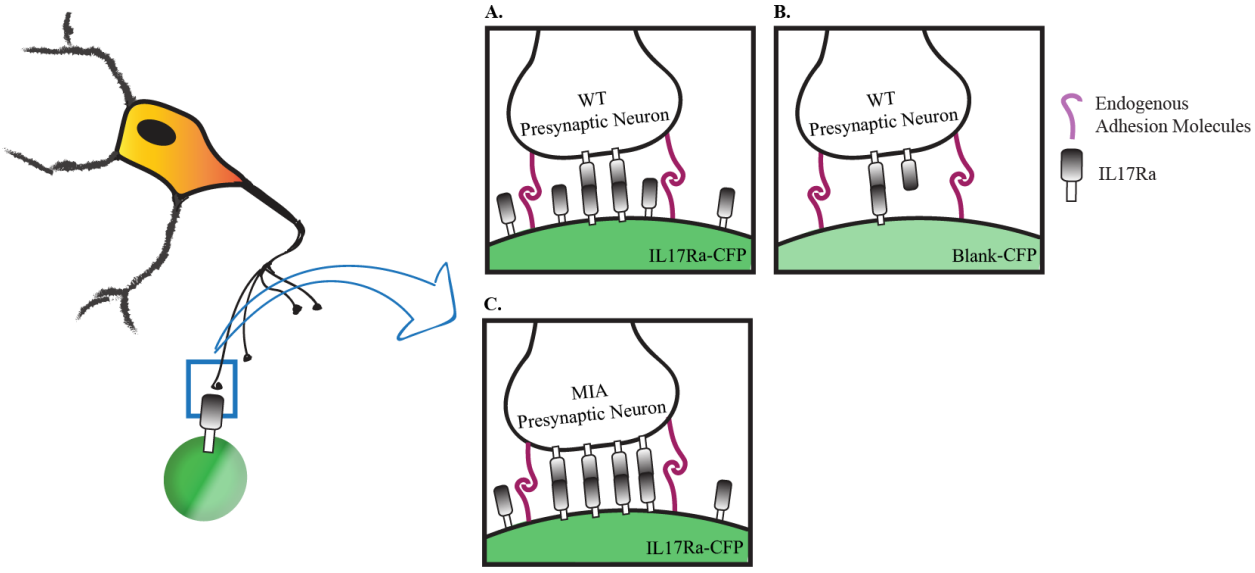


Figure 7: Hypothesized role of IL17Ra in the artificial synapse formation assay. (A) Wild type neurons with endogenous IL17Ra expression will exhibit certain level of synapse formation with IL17Ra-overexpressing non-neuronal cells. (B) If IL17Ra aids in synapse formation, in control situations, where the non-neuronal cells do not overexpress IL17Ra, synapse formation will be inhibited. (C) If IL17Ra aids in synapse formation, MIA neurons will exhibit increased synapse formation with IL17Ra-overexpressing non-neuronal cells, as MIA neurons overexpress IL17Ra as well.

CHAPTER 2: ROLE OF IL17Ra IN CELL ADHESION

Results

We investigated the role of the cytokine receptor IL17Ra in cell adhesion using the cell aggregation assay. We observed cell aggregation in HEK293 cells for the following reasons: (i) Endogenous IL17Ra expression levels in primary cortical neurons and HEK293 cells are similar (Fig. S1); and (ii) Non-neuronal cell adhesion assays are simple and easily reproducible, yet very effective tests to test the adhesive properties of a protein of interest.

This assay utilized HEK293 cells transfected with IL17Ra fused with CFP or YFP to assess whether overexpression of IL17Ra aids in cell adhesion. After transfection, the cells were treated with the protease trypsin, and the calcium chelator EDTA, to dissociate and inhibit endogenous cell adhesion molecules. The dissociated cells were then treated with calcium chloride to aid endogenous cell adhesion, and incubated in the presence or absence of the protein's ligand, IL-17. Finally, the cells were imaged at 0 hour, 2 hours, 4 hours, and 24 hours upon incubation to determine the changes in cell adhesion, represented by the area of the cell aggregate. As comparison, the same procedures were repeated using HEK293 cells transfected with different mock vectors fused with fluorescent proteins (BFP or mCherry) (Fig. 8). The adhesive properties of IL17Ra were characterized by measuring and comparing the size of cell aggregation in IL17Ra-transfected cells to that of mock-vector transfected cells via percent difference from baseline (0 Hr) (Fig. 9A).

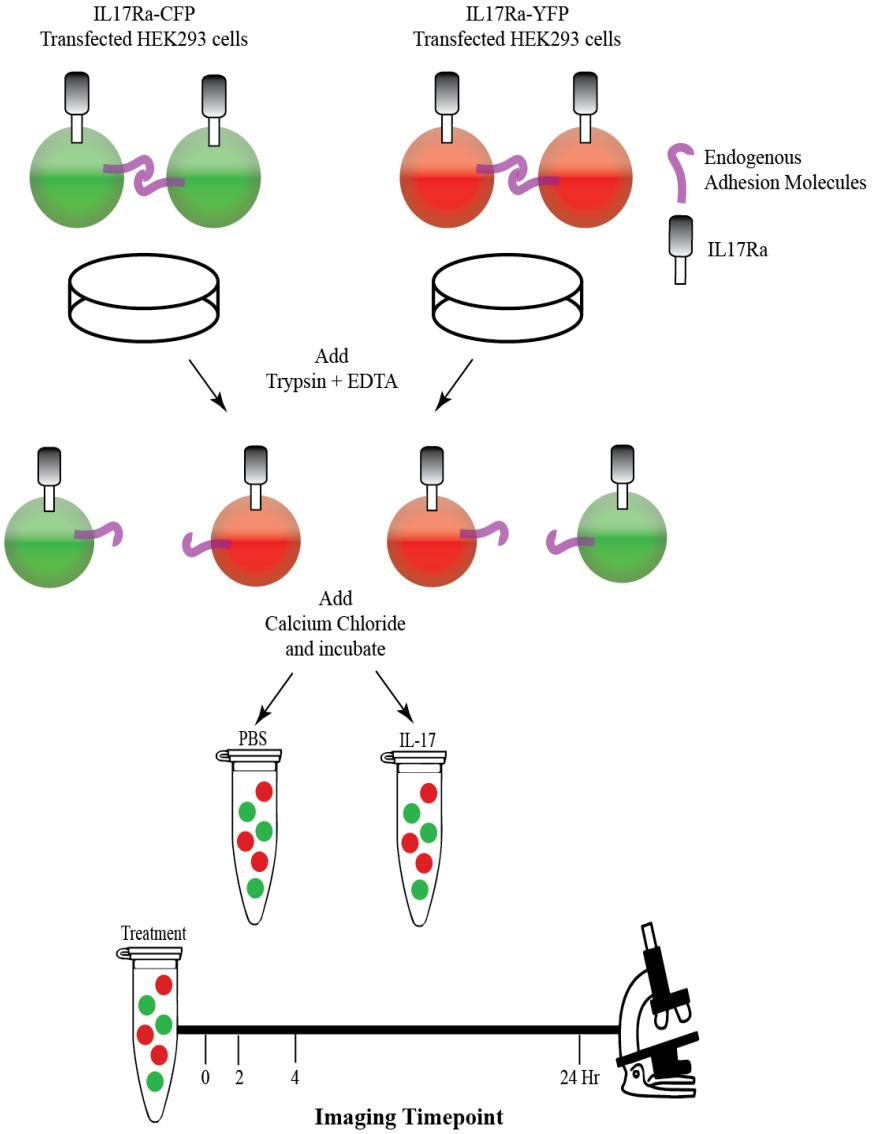


Figure 8: Schematic of cell aggregation assay protocol. HEK293 cells were transfected with two fluorescently labeled IL17Ra (CFP or YFP). Trypsin, a protease, and EDTA, a calcium chelator, were added to break apart and inhibit endogenous cell adhesion molecules. The cells were then treated with calcium chloride, and incubated with or without the ligand, IL-17. The cells were imaged at various time points to assess the role of IL17Ra in cell adhesion, as well as the effects of the presence of absence of ligand.

IL17Ra overexpression inhibits cell aggregation in the absence of ligand

In the absence of ligand (PBS treatment), both IL17Ra and blank-vector transfected cells showed time-dependent increases in cell aggregation (Fig. 9A). Quantification showed that IL17Ra overexpressing cells exhibited smaller % differences from baseline cell aggregation compared to those of blank-vector transfected controls after 2 hours, 4 hours, and 24 hours of incubation (Fig. 9B).

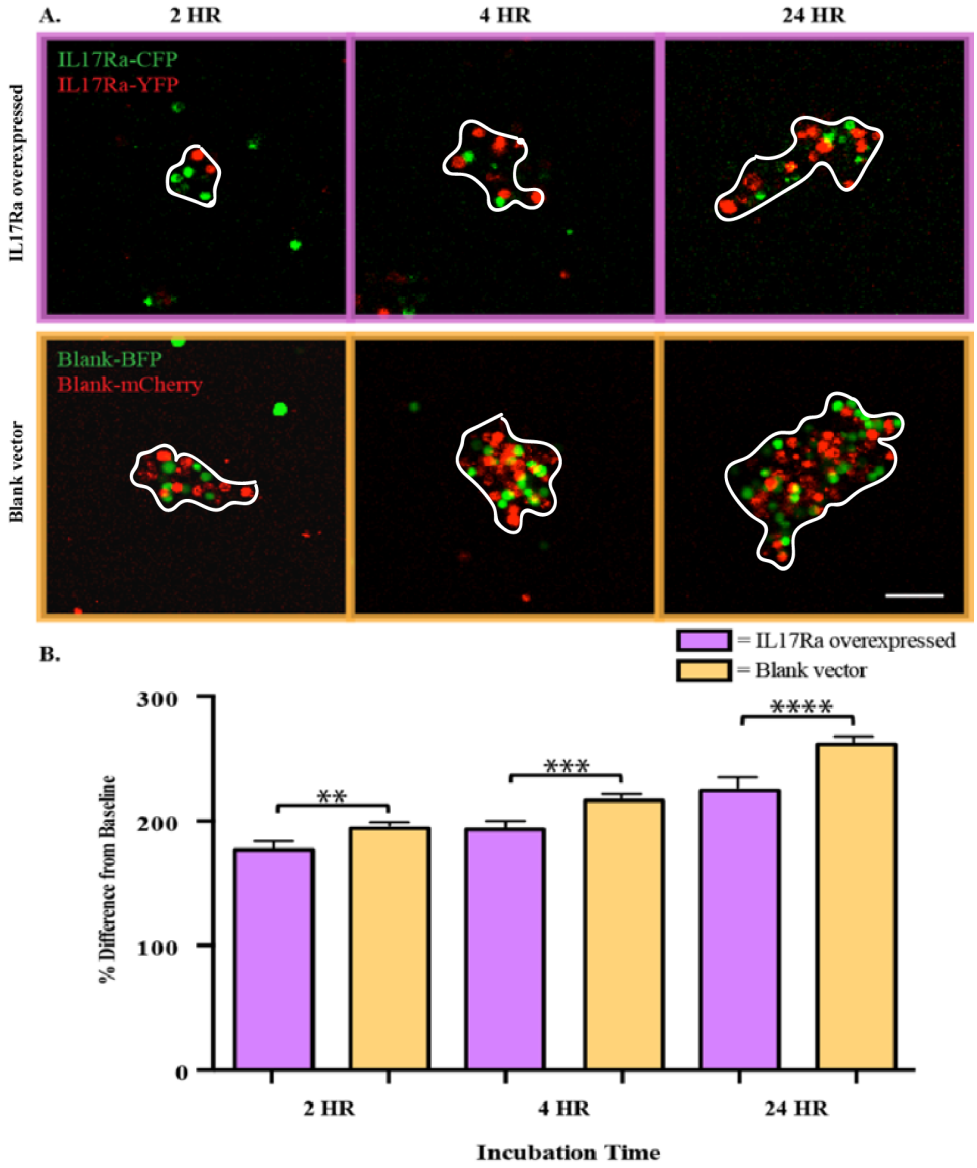


Figure 9: In the absence of ligand IL-17, overexpression of IL17Ra in HEK293 cells inhibits cell aggregation. The role of IL17Ra in cell adhesion was characterized by measuring the size of cell aggregation of HEK293 cells (outlined in white) transfected with IL17Ra (purple) or with blank vectors (orange) at various time points. (A) The percent difference from baseline (area of cell aggregates at 0 HR) was calculated to analyze the effect of IL17Ra in cell aggregation. Both groups of HEK293 cells incubated in the absence of ligands showed time-dependent increases in cell aggregation. B. Upon quantification, cells transfected with IL17Ra formed smaller cell aggregates than cells transfected with blank vectors at 2, 4, and 24 hours of incubation. All bars represent mean $\pm$ s.e.m. Unpaired t-test; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. All images were taken at 10X. Cell aggregate sizes were measured using ImageJ. Scale bar indicates 30µm.

Long-term treatment of IL-17 blocks the inhibition of cell aggregation in IL17Ra overexpressing cells

Both IL17Ra and blank-vector transfected cells showed time-dependent increases in cell aggregation in the presence of ligand (IL-17 treatment) (Fig. 10A). Similar to the cells incubated in the absence of ligand, IL17Ra overexpressing cells exhibited less cell aggregation compared to blank-vector transfected controls after 2 hours and 4 hours of incubation. However, after long-term (24 hours) exposure to IL-17, this inhibition of cell aggregation was blocked, and the two groups showed the same level of cell aggregation (Fig. 10B).

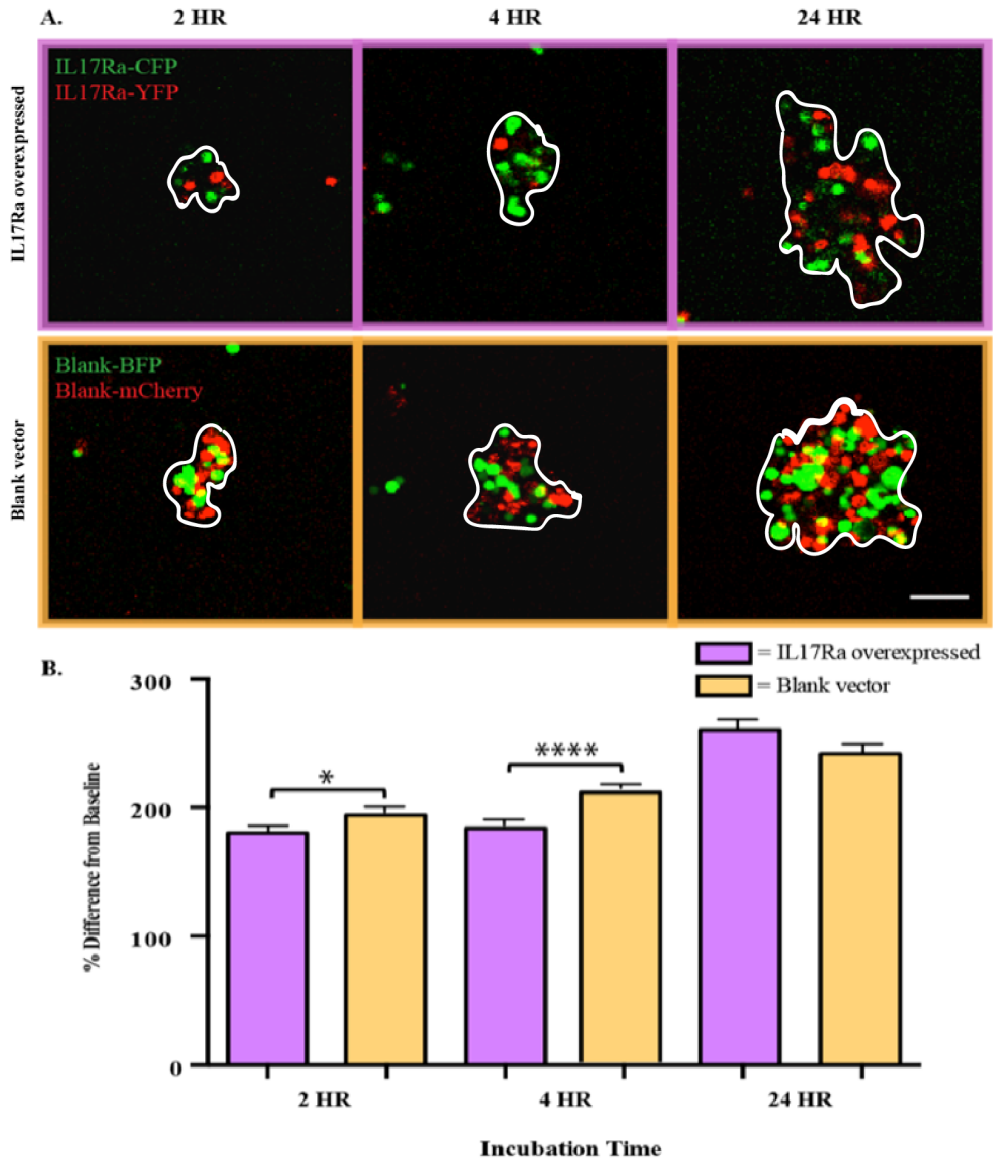


Figure 10: Long-term treatment of ligand IL-17 suppresses inhibition of cell aggregation. The role of IL17Ra in cell adhesion was characterized by measuring and comparing the size of cell aggregation of HEK293 cells (outlined in white) transfected with IL17Ra (purple) or mock (orange) vectors. (A) The percent difference from baseline (area of cell aggregates at 0 HR) was calculated to analyze the effect of IL17Ra expression in cell aggregation. Both groups of cells transfected with IL17Ra or with blank vectors, incubated in the presence of ligands, showed a time-dependent increase in cell aggregation. B. Upon quantification, IL17Ra overexpressing cells formed smaller aggregates than those transfected with blank vectors upon short-term incubation (2 hr and 4 hr). Upon long-term incubation of 24 hr, the two groups showed equal amount of cell aggregation, and inhibition of cell aggregation was blocked. All bars represent mean $\pm$ s.e.m. Unpaired t-test; *$p<0.05$, ****$p<0.0001$. All images were taken at 10X. Cell aggregate areas were measured using ImageJ. Scale bar indicates 30μm.

Inhibition of cell aggregation in IL17Ra overexpressing cells is blocked after long-term exposure to IL-17

IL17Ra overexpressing cells incubated in the absence or presence of ligand for four hours exhibited smaller aggregate sizes than control cells (Fig. 11A). This pattern of inhibition, however, was blocked upon long-term exposure to the ligand, and IL17Ra overexpressing and control cells exhibited similar levels of cell aggregation (Fig. 11B).

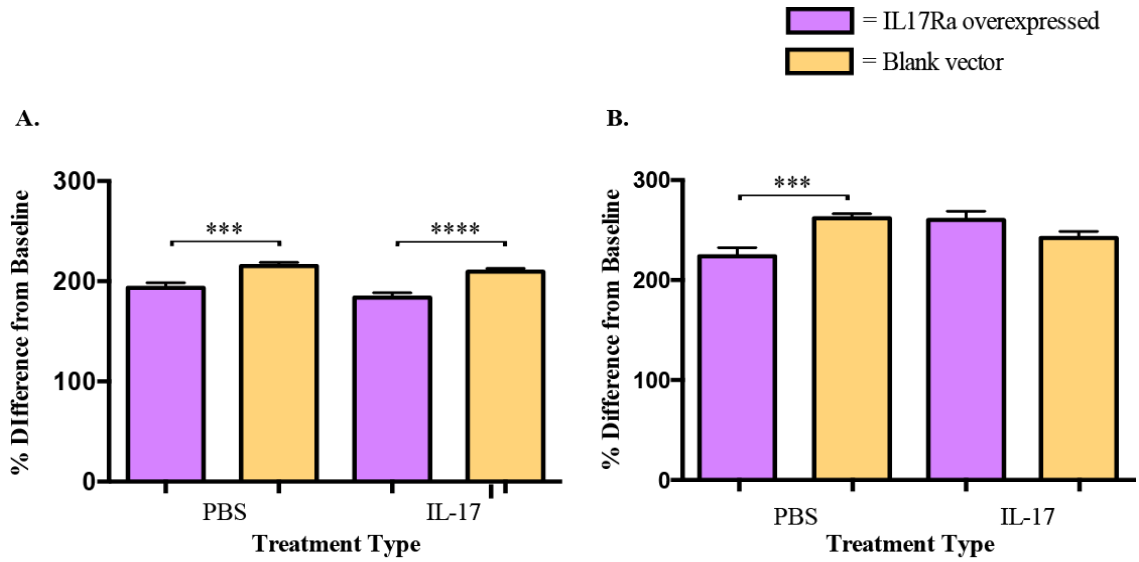


Figure 11: Long-term exposure to IL-17 suppresses inhibition of cell aggregation in IL17Ra overexpressing HEK293 cells. The role of IL17Ra in cell adhesion was characterized by measuring and comparing the size of cell aggregation of HEK293 cells transfected with IL17Ra (purple) or blank vectors (orange) in the absence or presence of ligand IL-17. (A) Upon 4 hours of incubation, IL17Ra expression inhibited cell aggregation, as shown by the decrease in cell aggregate size of IL17Ra overexpressing cells. (B). Upon 24 hours of incubation, PBS treated IL-17Ra overexpressing cells formed smaller aggregates compared to the aggregates observed among PBS treated control cells, IL-17 treatment, however, blocked this pattern of inhibition, and the two groups showed similar levels of aggregation. All bars represent mean $\pm$ s.e.m. One-way ANOVA; ***$p<0.001$, ****$p<0.0001$. Cell aggregate areas were measured using ImageJ.

Discussion

Maternal IL-6 concentrations increases upon inflammation, which results in increased IL-17 concentrations (*18, 33*). Although the mechanism is still unknown, maternal IL-17 is thought to travel to the fetus to induce increased expression of IL17Ra. The increased expression of IL17Ra then amplifies IL-17 signaling in the fetus, which adversely impacts neurodevelopment of the fetus and causes abnormal cortical and behavioral ASD-like phenotypes (*33*). Here we attempt to elucidate the role of IL17Ra during development by characterizing its adhesive properties. Our results from the cell aggregation assay indicate that IL17Ra acts as a protein with inhibitory roles in cell adhesion.

The purpose of the cell aggregation assay was to determine whether IL17Ra acts as a cell adhesion molecule to stimulate cell adhesion among HEK293 cells that overexpress the protein. Contrary to our hypothesis, our results indicated that IL17Ra expression in HEK293 cells inhibits cell adhesion. When IL17Ra expressing HEK293 cells were incubated in the absence of its ligand IL-17, lower levels of cell aggregation were observed compared to those of control cells (Fig. 9). On the other hand, when IL17Ra expressing HEK293 cells were incubated with its ligand IL-17, lower levels of cell aggregation were observed only upon short-term exposure to IL-17 (2 and 4 hr), and this inhibition was blocked upon long-term exposure (24 hr) (Fig. 10). Further analysis confirmed lower levels of cell aggregation in IL17Ra overexpressing cells incubated in the absence of ligand, and restored, control levels of cell aggregation in IL17Ra overexpressing cell incubated with its ligand (Fig. 11). Together, these results suggest that the expression of IL17Ra in HEK293 cells disrupts stable cell-cell interactions and inhibits cell aggregation.

One may argue that the lower levels of aggregation in IL17Ra are due to lower transfection efficiency of the IL17Ra, as well as the lower intensities of fluorescence. However, this can be accounted by the high sample size, as well as the high number of replicate studies. To further confirm our results, we will re-conduct this assay in a more controlled manner and normalize the assay to the number of single cells remaining instead. For example, one may argue that using cell aggregate area as a measure of cell aggregation is not very accurate because this method fails to account for the 3-dimensional aspect of cell aggregation. Instead, we will count the number of single cells remaining at each time point in our next set of experiments. In addition, since this cell aggregation assay was aimed towards defining IL17Ra's role as a potential cell adhesion molecule in aiding cell aggregation, but our results hint at a potential inhibitory role of IL17Ra, we will redesign the assay to test its inhibitory characteristics. For example, we will co-transfect non-neuronal cells with IL17Ra, as well as a well-characterized cell adhesion molecule, and compare the level of cell aggregation to that of cells transfected with IL17Ra only to observe whether the cells co-transfected with IL17Ra exhibit a pattern of inhibition in cell aggregation.

Based on our results, here we propose a model in which IL17Ra plays an inhibitory role in cell adhesion. IL17Ra has a binding partner, which upon binding destabilizes the interaction and leads to less cell adhesion (Fig. 12B). Furthermore, this model also explains why the introduction of IL-17 can block the inhibition that occurs in the presence of IL17Ra. In the presence of its ligand, IL17Ra will bind to IL-17 instead, and therefore block the inhibition of cell adhesion that normally occurs in the absence of its ligand (Fig. 12C).

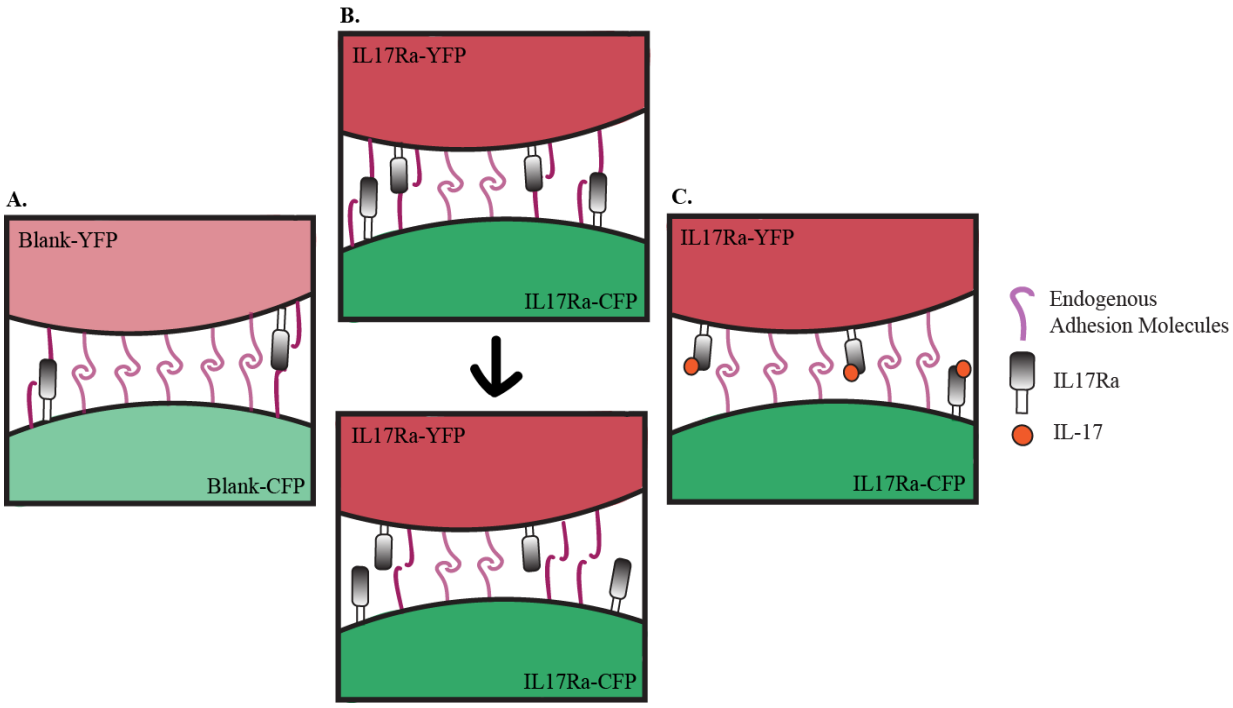


Figure 12: A proposed model for the role of IL17Ra in cell adhesion. (A) When control non-neuronal cells are incubated in the absence of ligand, the cells will exhibit a certain level of cell adhesion. (B) When non-neuronal cells overexpressing IL17Ra are incubated in the absence of ligand, IL17Ra will bind to other cell adhesion molecules to destabilize the interaction between the cells. (C) However, when the ligand is introduced, IL17Ra has a higher affinity to its ligand than to other endogenous cell adhesion molecules, therefore blocking inhibition that occurred in the absence of ligand.

This now raises the question, whether or not IL17Ra has other binding partners to mediate changes in cell adhesion, as well as what specific domain of the protein is responsible for bringing about these changes. Future studies will include co-transfecting the cells with a well-characterized cell adhesion molecule and IL17Ra to confirm inhibition in cell aggregation, using pull-down assays to isolate the binding partners of IL17Ra, using mass spectrometry to identify the binding partners, as well as transfecting truncated forms of IL17Ra to determine the domain responsible for inhibitory changes. Although cell aggregation assay uses HEK293 cells and therefore is not directly translatable to neurodevelopment, this model allows for identifying independent contributions of IL17Ra in cell adhesion. Furthermore, HEK293 cells are very

stable cell lines that allow us to easily renew and reproduce more cells for future studies. With results from this assay, we may soon be able to accurately confirm whether or not IL17Ra has inhibitory functions, and better design other assays to characterize the role of IL17Ra.

CHAPTER 3: ROLE OF IL17Ra IN NEURITE OUTGROWTH

Results

We next examined the role of cytokine receptor IL17Ra in the morphogenesis of wild type cortical neurons *in vitro* by analyzing their neurite development. We observed neurite outgrowth in primary cortical neurons for the following reasons: (i) *in vitro* experiments allow greater control of environment and manipulation of genome; (ii) Cell adhesion molecules are known to regulate morphogenesis of cortical neurons; (iii) Abnormal corticogenesis has been implicated in many MIA models, as well as in human studies (*33, 44*).

In order to test this, we compared the neurite outgrowth lengths in neurons with endogenous IL17Ra expression and with IL17Ra KD to measure the effect of IL17Ra loss-of-function. In preparation of the primary neuronal morphology assay, wild type primary cortical neurons were plated, and at days in vitro (D.I.V.) 4, LacZ or IL17Ra was knocked-down using the CRISPR/Cas9 technique. LacZ (endogenous IL17Ra expression levels) or IL17Ra (IL17Ra loss-of-function) knockdown was confirmed visually, as Cas9 transduction was indicated by GFP fluorescence, and via SURVEYOR nuclease assay (Fig. S2). Furthermore, these two groups were grown either in the absence (PBS treatment) or the presence of ligand (IL-17 treatment), and fixed and imaged at D.I.V.13. (Fig. 13A). The images taken at D.I.V. 13 were then analyzed for neurite outgrowth by measuring the neurite length between two cell bodies (Fig. 13B).

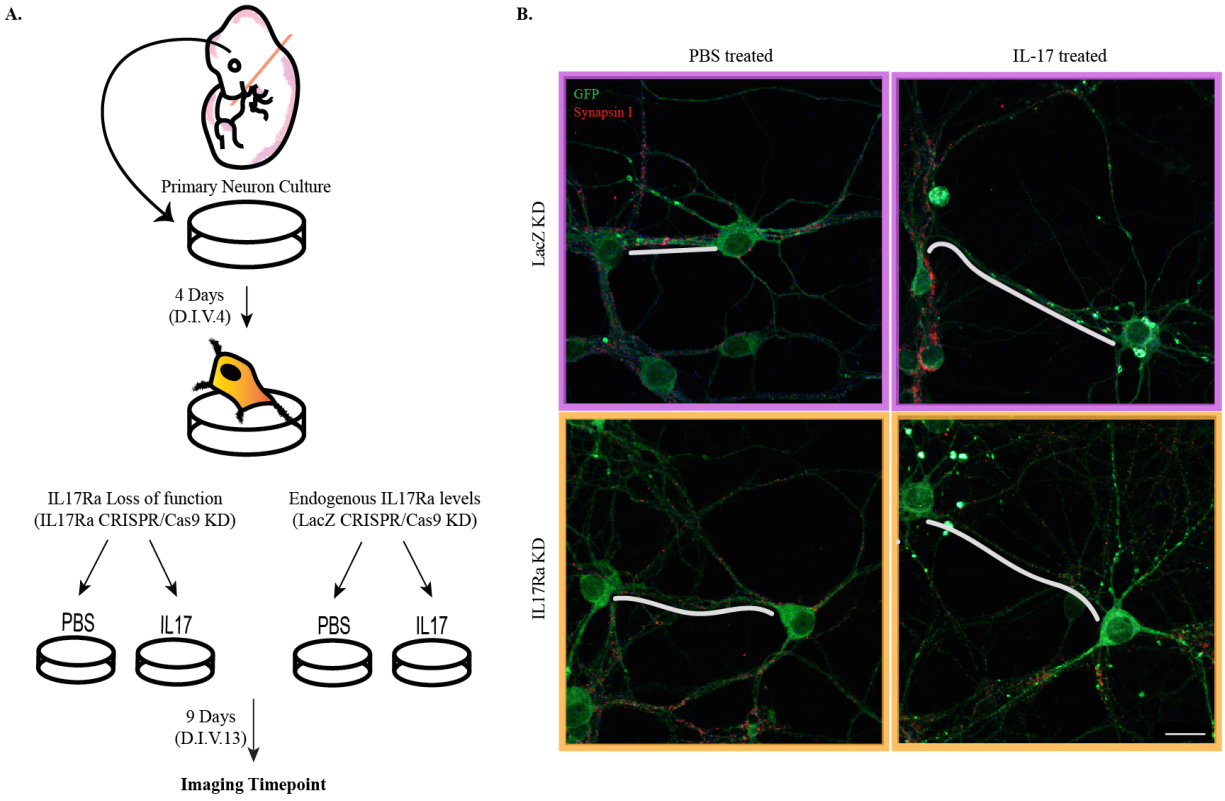


Figure 13: Schematic of morphology assay protocol. (A) Primary cortical neurons from wild type animals were plated and grown for 4 days. At days in vitro (D.I.V.) 4, the genomes of the cells were edited using the CRISPR/Cas9 editing technique. Then, the cells were grown in the absence (PBS treatment) or the presence (IL-17 treatment) for 9 more days. Cells were fixed at D.I.V. 13 and stained. (B) The imaged cells were analyzed for neurite outgrowth using ImageJ. Neurite length between two cell bodies was measured for each group and treatment type, and the mean values were compared. All images were taken at 30X magnification. Scale bar is indicative of 50µm.

Treatment of ligand IL-17 increases neurite outgrowth in wild type primary cortical neurons

In wild type primary cortical neurons with LacZ KD, treatment with IL-17 resulted in approximately 1.8 times longer neurite outgrowth than those grown in the absence of ligand (Fig. 14A). Similarly, treatment of IL-17 resulted in approximately 1.6 times longer neurite outgrowth in wild type primary cortical neurons with IL17Ra KD than those grown in the absence of ligand (Fig. 14B).

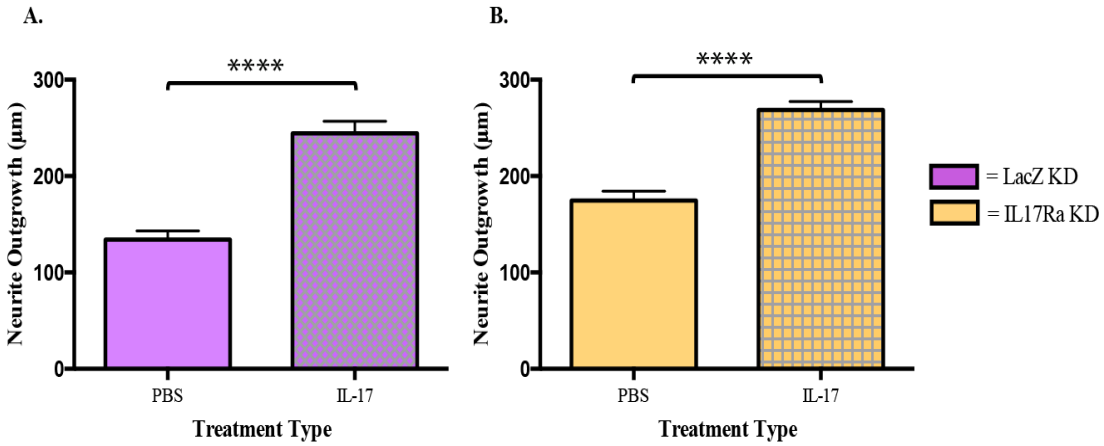


Figure 14: Treatment of ligand IL-17 increases neurite outgrowth in wild type primary cortical neurons. Neurite outgrowth of wild type primary cortical neurons with LacZ KD (purple) or IL17Ra KD (orange) was measured. (A) IL-17 treated LacZ KD neurons showed longer neurite outgrowth than control neurons. (B) Similarly, IL-17 treated IL17Ra KD neurons showed longer neurite outgrowth than control neurons. All bars represent mean $\pm$ s.e.m. Unpaired t-test; **** $p < 0.0001$.

IL17Ra loss-of-function in wild type primary cortical neurons increases neurite outgrowth

Quantification showed that IL17Ra neurons grown in the absence of IL-17 exhibit approximately 1.3 times longer neurites than LacZ neurons grown in similar conditions (Fig. 15A). Although treatment of IL-17 resulted in increases in neurite outgrowth in both LacZ KD and IL17Ra KD neurons, this increase was further amplified in the IL17Ra KD group (Fig. 15B).

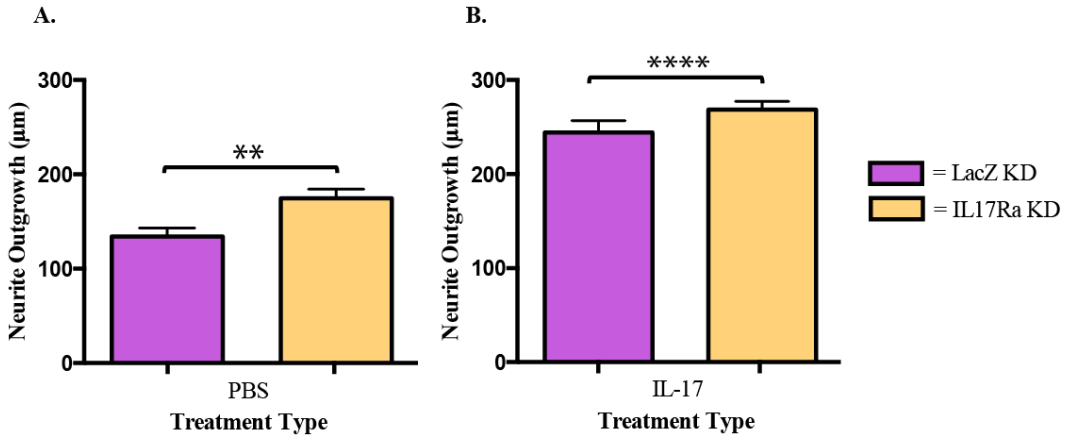


Figure 15: IL17Ra loss-of-function results in increased neurite outgrowth. Neurite outgrowth of wild type primary cortical neurons with LacZ KD (purple) or IL17Ra KD (orange) was measured. (A) Neurons with IL17Ra loss-of-function showed increased neurite outgrowth in the absence of ligand. (B) Neurons with IL17Ra loss-of-function showed further increase in neurite outgrowth upon IL-17 treatment, compared to neurons with endogenous IL17Ra expression. All bars represent mean $\pm$ s.e.m. Unpaired t-test; **p<0.01, ****p <0.0001.

Discussion

The purpose of the primary cultured neurite outgrowth assay was to determine the effects of IL17Ra loss-of-function on the morphogenesis, specifically on neurite outgrowth, of primary cortical neurons. Contrary to our hypothesis that the cytokine will only induce growth in control neurons with endogenous IL17Ra expression, both control and IL17Ra KD groups exhibited increases in neurite outgrowth when treated with cytokine IL-17 (Fig. 14). Furthermore, contrary to our hypothesis that IL17Ra loss-of-function will suppress neurite outgrowth, neurite outgrowth increased in both endogenous IL17Ra expressing and IL17Ra KD neurons, and this growth was further amplified in the IL17Ra KD group (Fig. 15). Together, these results show that cytokine IL-17 can induce neurite outgrowth even in the absence of its receptors. Furthermore, these results suggest that expression of IL17Ra leads to inhibition of neurite outgrowth, and upon KD, this inhibition is blocked to further induce neurite outgrowth. Although the neurotrophin-like role of IL-17 was unexpected, the inhibitory pattern of IL17Ra is consistent with our results from the cell aggregation assay.

One may raise the question of reliability in knocking-down IL17Ra using the CRISPR/Cas9 genome editing technique. However, we have confirmed CRISPR/Cas9 KD efficiency using the SURVEYOR nuclease assay (Fig. S2), and also recently have generated a KO murine line to repeat and confirm our results. Future studies will involve using primary cortical neurons from IL17Ra KO mice instead of those with CRISPR/Cas9 KD to assure attribute results from our assays to IL17Ra loss-of-function. Furthermore, our result that neurite outgrowth was increased even in IL17Ra KD neurons hints at the possibility of the Th17/IL-17 signaling pathway having normal physiological functions during brain development. Further supporting this notion, previous studies have observed structural similarities between the family

of IL-17 cytokines and neurotrophic factors (e.g. nerve growth factor) (*45, 46*). Interestingly, these results suggest the possibility of cross-talk between the IL-17 and neurotrophic factor signaling pathways to mediate normal development. On the same note, during normal cortical development, it is important for the axons to be able to adhere to various classes of adhesion molecules. It is also crucial to be able to detach, instead of adhere, by downregulating and/or inhibiting of cell adhesion molecules. For example, changes in cell adhesion molecule activity are critical in order to pioneer new directions of neurite outgrowth and arrive at the desired location (*47*). In order to further elucidate the mechanism by which IL17Ra facilitates neurite outgrowth and axonal pathfinding, we will conduct a neuronal migration assay that utilizes tissue sections and their neuronal migration patterns to examine the effects of IL-17 signaling on development.

Previous findings have shown that MIA disrupts cortical development (*33, 48*). Based on our findings, here we propose a mechanism by which abnormal neurite outgrowth may occur. During axonal pathfinding, the inhibition of a set of cell adhesion molecules is required in order to change directions (*40*). IL17Ra may play an inhibitory role and downregulate cell adhesion to the extracellular matrix to suppress neurite outgrowth (Fig. 16A). In neurons with IL17Ra KD, this inhibition is blocked, and we may observe increases in cell motility and neurite outgrowth (Fig. 16B). On a similar note, IL17Ra may play an inhibitory role and suppress cell adhesion molecules to guide axonal pathfinding during directional changes (Fig. 16C). On the other hand, when IL17Ra is overexpressed in the MIA model, too much inhibition may result in its inability to reach the desired destination, and abnormal/ overshoot of the neurite (Fig. 16D). It is important to keep in mind that this *in vitro* assay has limitations, as it is not fully representative of conditions *in vivo*. However, the advantages outweigh its limitations, as this *in vitro* model

allows us to identify independent contributions of IL17Ra in morphogenesis. With more experiments, we may be able to elucidate the mechanisms by which dysregulation of the IL17Ra signaling mechanism induces abnormal cortical development.

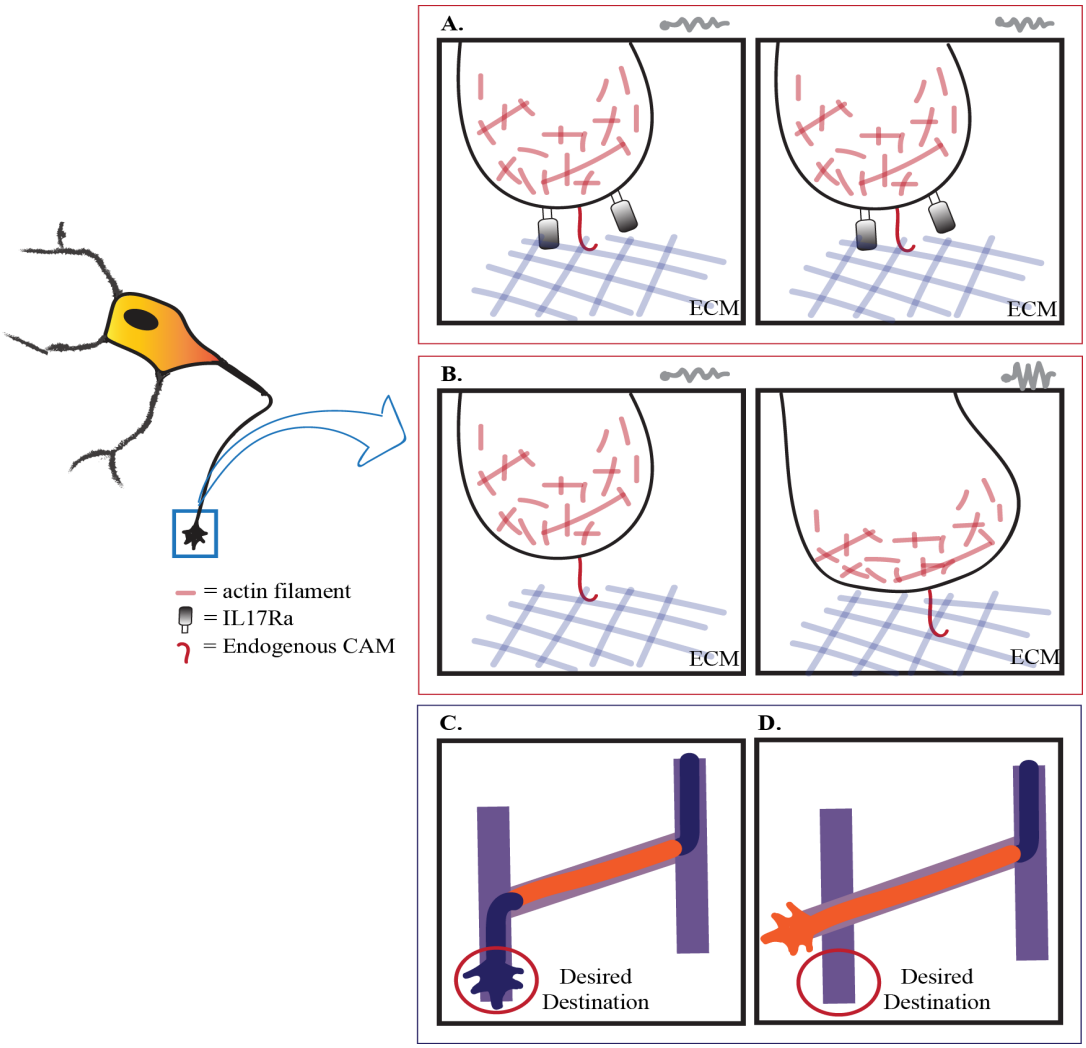


Figure 16: A proposed model for the role of IL17Ra during neurite outgrowth and axon pathfinding. (A) IL17Ra normally inhibits adhesion to the extracellular matrix (ECM) that is required for neurite outgrowth. (B) Upon IL17RA loss-of-function, this inhibition is blocked, and neurite outgrowth increases. **(**C) During normal development, one set of cell adhesion molecules are needed to guide the axons to grow in a certain direction (navy). Inhibition of this set of cell adhesion molecules is needed (orange) via IL17Ra signaling, in order to detach and guide the axon towards the desired destination (red circle). (D) Upon IL17Ra loss-of-function, and therefore disruption of normal neurite outgrowth and axon pathfiding, the axon will continue growing, not only resulting in increased neurite outgrowth, but also abnormal cortical development.

CHAPTER 4: ROLE OF IL17Ra IN SYNAPSE FORMATION

Results

In order to investigate the role of cytokine receptor IL17Ra in synapse formation, the puncta formation development in wild type primary cortical neurons was analyzed *in vitro* as another measure of morphogenesis.

Specifically, we compared the synapse puncta count in neurons with endogenous IL17Ra expression and with IL17Ra KD to measure the effects of IL17Ra loss-of-function. Wild type primary cortical neurons were plated, and at days in vitro (D.I.V.) 4, the genomes of the cells were edited using the CRISPR/Cas9 technique. More specifically, the groups of comparison were primary cortical neurons with LacZ KD (endogenous IL17Ra expression) or IL17Ra KD (IL17Ra loss-of-function). Furthermore, these two groups of neurons were grown in the absence (PBS treatment) or the presence of ligand (IL-17 treatment), and fixed and imaged at D.I.V.13 (Fig. 17A). The images taken at D.I.V.13 were analyzed for synapse puncta development by comparing the synapse puncta count on 50μm of the neurites in various groups (Fig. 17B).

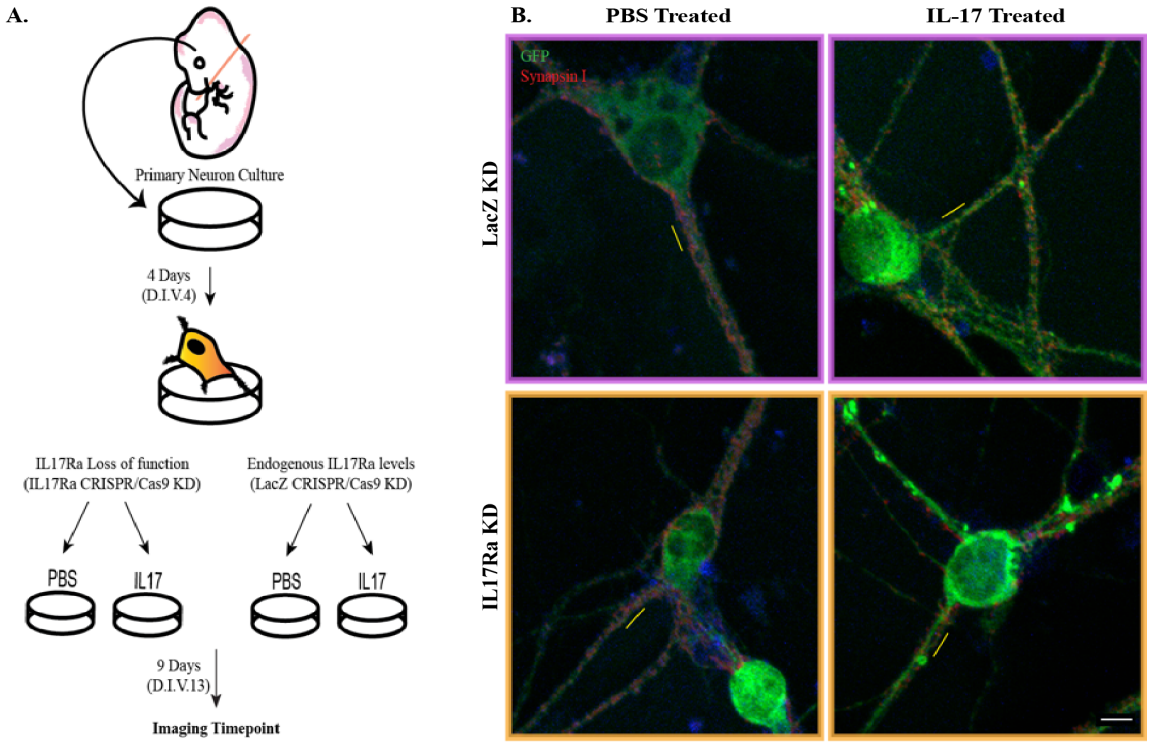


Figure 17: Schematic of synapse puncta count assay protocol. (A) Primary cortical neurons were plated and grown for 4 days. At days in vitro (D.I.V.) 4, the genomes of the cells were edited by cleaving the sequences of IL17Ra or LacZ via the CRISPR/Cas9 technique. Guide RNA's were fused with GFP. The cells were then grown for 9 more days at the absence of (PBS treatment) or the presence of ligand (IL-17 treatment). At D.I.V.13, all cells were fixed ad imaged. (B) The imaged cells were then analyzed for synapse puncta formation via the software ImageJ. All synapse puncta on the neurites, represented by synapsin I staining (red), 50µm away from the cell body were counted for 50µm (yellow line). Then, the synapse puncta count of various groups and treatment types were compared. All cells were stained for GFP (green) and synapsin I (red). All images were taken at 40X magnification. Scale bar indicates 30µm.

Treatment of ligand IL-17 suppresses synapse puncta formation in wild type primary cortical neurons upon IL17Ra loss-of-function

In wild type primary cortical neurons with LacZ KD, there was no difference in synapse puncta count between PBS and IL-17 treated neurons (Fig. 18A). Surprisingly, upon IL17Ra KD, treatment of IL-17 induced differences in synapse puncta count. Quantification showed that neurons treated with IL-17 had about 1.4 times less synapse puncta formation than those grown without (Fig. 18B).

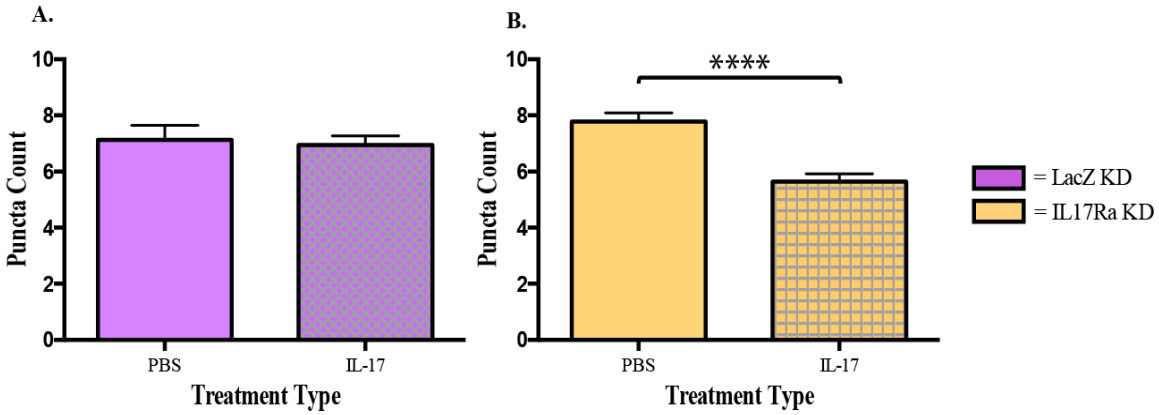


Figure 18: Treatment of ligand has no effect on LacZ KD wild type primary cortical neurons, but suppresses puncta formation in IL17Ra KD neurons. Synapse puncta formation in wild type primary cortical neurons with LacZ KD (purple) or IL17Ra KD (orange) was measured. (A) LacZ KD neurons showed the same level of synapse puncta formation when cultured in the absence (no pattern) or presence of IL-17 (patterned). (B) However, IL-17 treatment suppressed synapse formation in IL17Ra KD neurons (patterned) compared to that of control neurons (no pattern). All bars represent mean $\pm$ s.e.m. Unpaired t-test; **$p<0.01$.

IL17Ra loss-of-function in wild type primary cortical neurons suppresses puncta formation in the presence of ligand IL-17

Upon quantification, the wildtype neurons that expressed endogenous levels of IL17Ra (LacZ KD) and those that underexpressed IL17Ra (IL17Ra KD) developed the same number of puncta at D.I.V. 13, when grown in the absence of ligand (Fig. 19A). In the presence of ligand, synapse puncta formation was suppressed when IL17Ra expression was reduced (Fig. 19B).

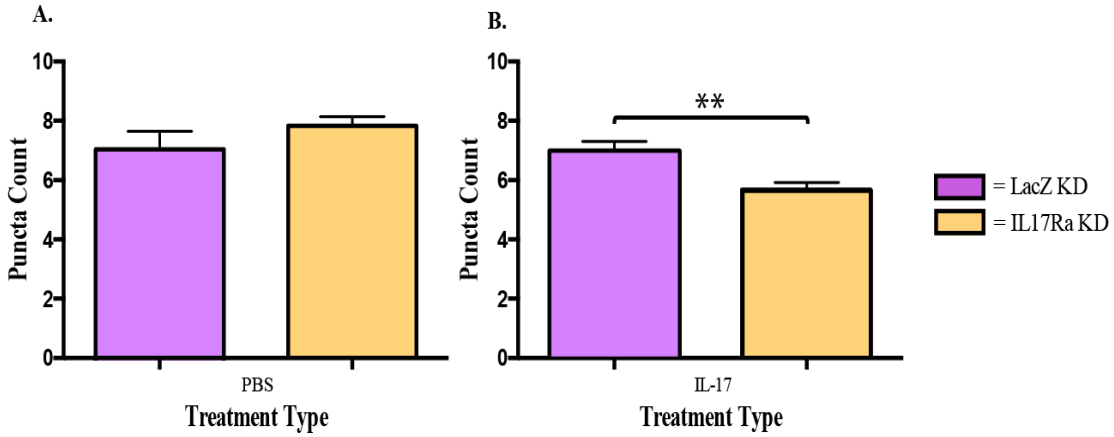


Figure 19: IL17Ra loss-of-function in wildtype primary cortical neurons inhibits synapse puncta formation when treated with ligand. The role of IL17Ra in synapse puncta formation was studied using primary cortical neurons with endogenous IL17Ra (LacZ KD) and with IL17Ra loss-of-function (IL17Ra KD). (A) Neurons with LacZ KD (purple) or IL17Ra KD (orange) did not differ in synapse puncta count when grown in the absence of ligand. (B) The presence of ligand suppressed synapse puncta formation in IL17Ra KD neurons. All bars represent mean $\pm$ s.e.m. ** $p<0.01$.

Overexpression of IL17Ra in non-neuronal cells inhibits synapse formation

In addition to the synapse puncta assay, we attempted to characterize the role of IL17Ra in synapse formation using the artificial synapse assay. We observed synapse formation between primary cortical neurons and HEK293 cells for the following reasons: (i) Cell adhesion molecules that are transfected onto non-neuronal cells are known to form stable junctions with neuronal cells; (ii) Previous studies show that this interaction between neuronal and non-neuronal cells induces similar differentiation processes that occur between neuronal cells upon synapse formation; (iii) Directly transfecting proteins onto neuronal cells is much more difficult to do because primary neuronal cultures are very sensitive to environmental changes; and (iv) Results from the artificial synapse formation assay will provide a functional readout of the synaptic adhesive properties of IL17Ra (*49, 50*).

In this assay, non-neuronal HEK293 cells overexpressing IL17Ra (transfected with IL17Ra-CFP) or endogenous levels of IL17Ra (transfected with blank-BFP) were seeded onto primary cortical neurons of wild type (WT) or maternal immune activated (MIA) animals. The cells were co-cultured for 2 days, and fixed and stained via immunocytochemistry using antibodies that targeted the presynaptic protein synapsin I (Fig. 20A). Synapse formation was then quantified by measuring the intensity of synapsin I expression, in the areas that co-localized with IL17Ra-CFP or blank-BFP expression (Fig. 20B).

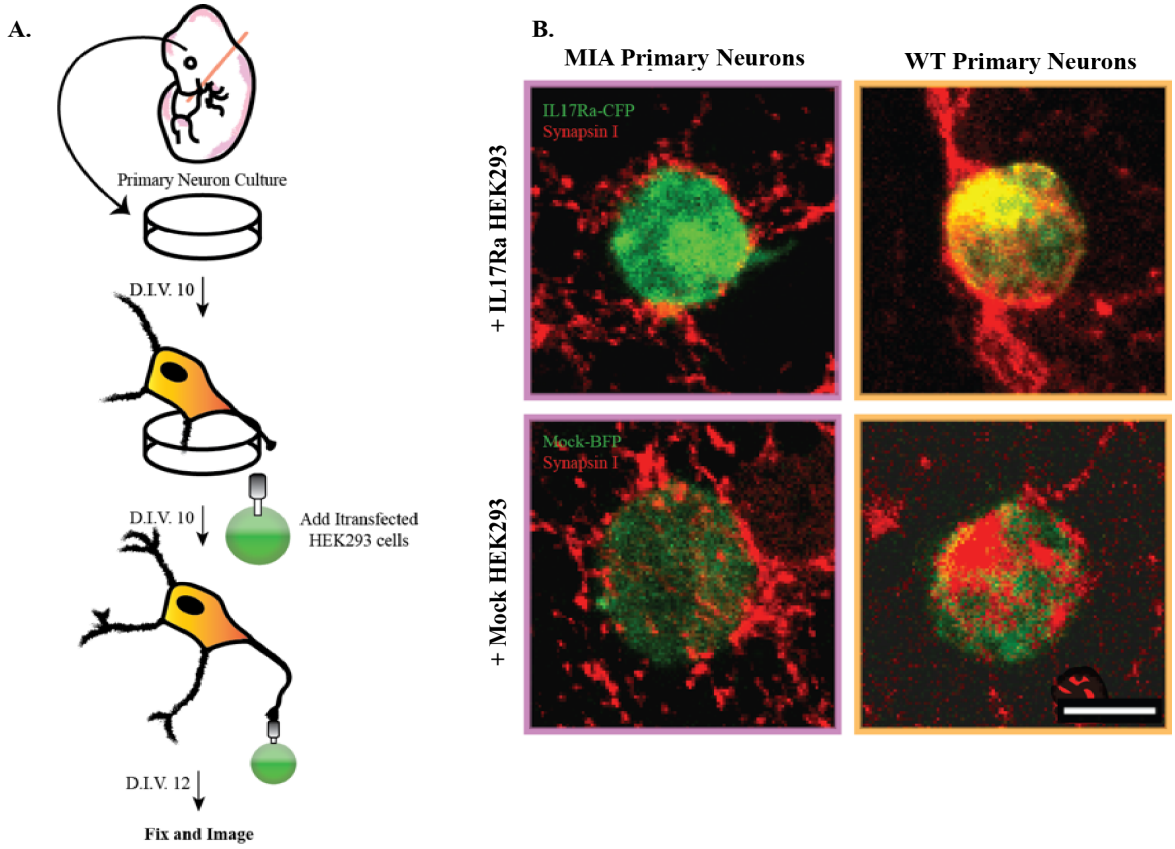


Figure 20: Schematic of artificial synapse formation assay protocol. (A) Primary cortical neurons from maternal immune activated (MIA) and WT animals were plated and grown for 10 days. At days in vitro (D.I.V.) 10, HEK293 cells transfected with IL17Ra or mock vectors were added. Cells were then fixed at D.I.V.12 and stained. (B) The imaged cells were analyzed for artificial synapse formation using the software program ImageJ. Synapse formation, represented by synapsin I intensity (red), was measured by analyzing the co-localization of transfected HEK293 cells and synapsin I staining. All images were taken at 40X magnification. Scale bar is indicative of 30µm.

Wild type primary cortical neurons co-cultured with non-neuronal cells overexpressing IL17Ra visibly showed inhibited synapse formation compared to those co-cultured with control cells alone (Fig. 20B). In MIA neurons, synapse formation was inhibited when co-cultured with non-neuronal transfected with blank vectors, and this inhibition was amplified when co-cultured with non-neuronal cells expressing IL17Ra (Fig. 20B).

Quantification showed that MIA neurons co-cultured with IL17Ra-overexpressing non-neuronal cells formed about 3 times fewer synapses than those co-cultured with control non-neuronal cells (Fig. 21A). Furthermore, wild type neurons co-cultured with IL17Ra-overexpressing non-neuronal cells exhibited slightly inhibited, but insignificant, synapse formation compared to those co-cultured with control non-neuronal cells (Fig 21B).

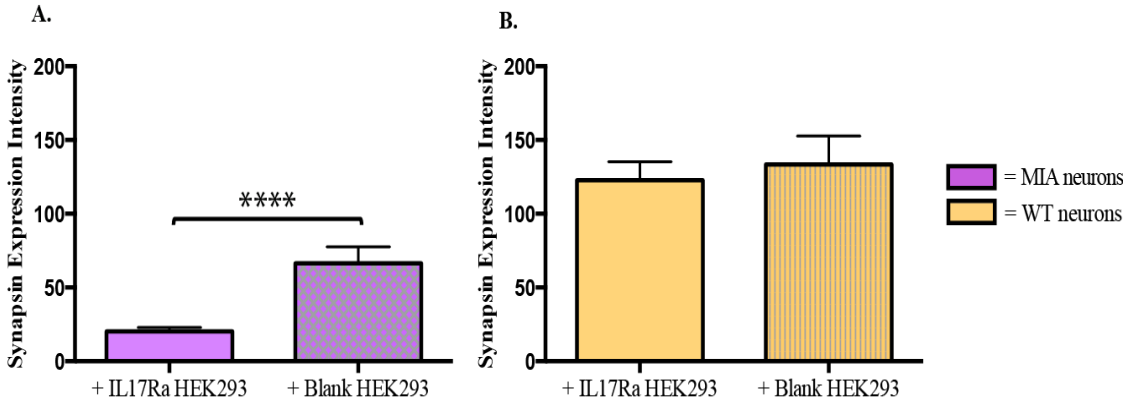


Figure 21: Synapse formation is inhibited when IL17Ra is overexpressed in non-neuronal cells. Non-neuronal cells transfected with IL17Ra or blank vectors were co-cultured with primary cortical neurons from MIA (purple) or wild type (orange) animals to determine the role of IL17Ra in synapse formation. (A) MIA neurons co-cultured with IL17Ra-overexpressing HEK293 cells resulted in 3 times less synapse formation than those co-cultured with blank HEK293 cells. (B) WT neurons co-cultured with IL17Ra-overexpressing showed slightly inhibited, but non-significant, synapse formation compared to those co-cultured with mock vector transfected cells. All bars represent mean $\pm$ s.e.m. ****$p<0.0001$.

Synapse formation is further inhibited in MIA primary cortical neurons

In addition to comparing the effects in synapse formation when IL17Ra is overexpressed in non-neural cells, we observed the effects of animal type. The general trend of inhibition in synapse formation when IL17Ra is overexpressed in non-neural cells was similar between the two groups, wild type and MIA neurons. However, MIA animals was further impaired in their abilities to form synapses, in comparison to those of wild type neurons (Fig. 22).

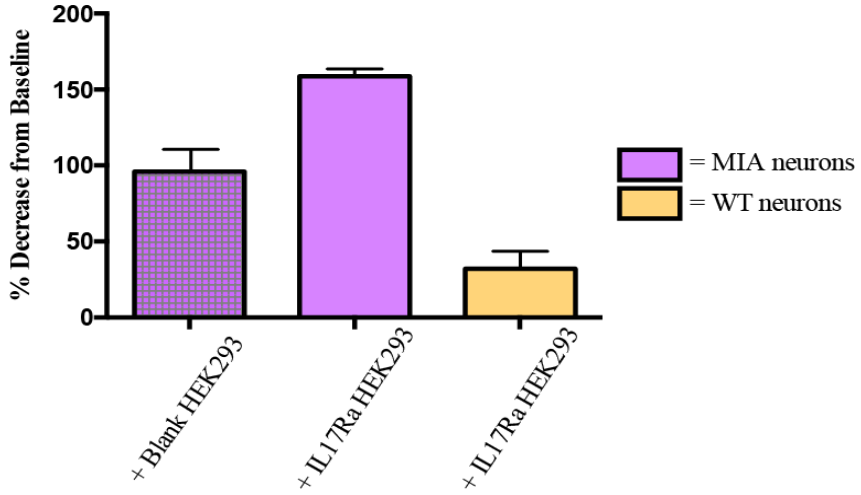


Figure 22: Synapse formation in maternal immune activated (MIA) primary cortical neurons is impaired. Non-neuronal cells transfected with IL17Ra or blank vectors were co-cultured with primary cortical neurons from MIA (purple) or wild type (orange) animals to determine the role of IL17Ra in synapse formation. IL17Ra expression in non-neuronal cells impaired synapse formation in wild type neurons slightly, as shown by the % decrease in baseline synapse formation between wild type neurons and control cells. This pattern is also observed in MIA neurons as well; however the inhibitory effects are much more amplified. All bars represent mean $\pm$ s.e.m.

Discussion

The purpose of the synapse puncta and artificial synapse formation assays were to characterize the role of IL17Ra as a cell adhesion molecule that facilitates synapse formation in primary cortical neurons. Contrary to our hypothesis that IL17Ra will aid synapse formation, thus decreasing synapse puncta formation upon loss-of-function and increasing artificial synapse formation with IL17Ra-overexpressing HEK293 cells, our results indicate that IL17Ra inhibits synapse formation.

In the synapse puncta assay, there was no difference in synapse puncta count between control and IL17Ra KD neurons when treated with PBS; however, when they were treated with IL-17, IL17Ra KD neurons developed less synapse puncta (Fig. 19). These results may be because the largest increase in neurite outgrowth was observed in IL-17 treated IL17Ra KD neurons, and therefore the synapse puncta may have been more spread out. Furthermore, we recognize the limitations of counting the number of synapse puncta for only 50μm of the neurites. Future studies will involve normalizing the each assay by measuring the synaptic density per neurite length in order to make more definitive conclusions regarding synapse puncta formation.

In the artificial synapse formation assay, synapse formation between MIA primary cortical neurons and blank HEK293 cells was greatly inhibited, and even further inhibited when the HEK293 cells overexpressed IL17Ra (Fig. 21A). Although a slight inhibition in synapse formation was observed between wild type primary cortical neurons and IL17Ra overexpressing HEK293 cells, the level of synapse formation did not differ significantly from that of wild type neurons and blank HEK293 cells (Fig. 21B). These results suggest IL17Ra has an inhibitory role in cell adhesion, and therefore suppresses synapse formation as well. Additionally, MIA neurons overexpress IL17Ra, further supporting this notion (*33*).

One may question whether or not synapsin I intensity is indicative of synapse formation. Although more accurately described as measuring contact between the neuronal and non-neuronal cells, many previous studies have confirmed that the differentiation processes that occur upon contact is representative of synapse formation that occurs between neuronal cells (*41, 49, 50*). Furthermore, our results from the artificial synapse formation assay are consistent with our findings from the cell aggregation and neurite outgrowth assays. Cell adhesion is a crucial element in synapse formation (*42*), and if IL17Ra has an inhibitory role, it is logical that synapse formation would also be inhibited in IL17Ra-overexpressing cells. However, future studies are needed to further elucidate the role and mechanism of IL17Ra in synapse formation. Although more difficult to do, analyzing synapse formation between cortical neurons via transfecting IL17ra onto neurons would yield more meaningful and translatable results. Lastly, confirming our results from the synapse puncta and artificial synapse assays using primary cortical neurons of IL17Ra knockout mice to measure the effects of complete IL17Ra loss-of-function would strengthen our hypothesis.

Putting our results into larger context, proper regulation of cell adhesion molecules is extremely critical, as cell adhesion molecules have central roles during the processes of cell adhesion, axon fasciculation, cell motility and neurite outgrowth, and synaptogenesis in neurodevelopment (*42*). However, the downregulation of these molecules is equally important, especially because these processes require some degree of inhibition in order for the neurons to detach, change directions, and prune synapses (*40*). Based on our results, here we propose a mechanism that involves IL17Ra in synapse formation. Wild type neurons form a stable level of connection via other endogenous cell adhesion molecules (Fig. 23A). This stable level of synapse formation is slightly disrupted when the non-neuronal cells overexpress IL17Ra, as

IL17Ra plays an inhibitory role (Fig. 23B). However, in MIA neurons that overexpress IL17Ra, this inhibitory effect is amplified (Fig. 23C), and even further amplified when the non-neuronal cells overexpress IL17Ra as well (Fig. 23D). Again, it is important to recognize the limitations, as well as the advantages, of *in vitro* assays. With further well-designed and well-controlled studies, we may soon be able to accurately characterize the role of IL17ra in the pathogenesis of ASD.

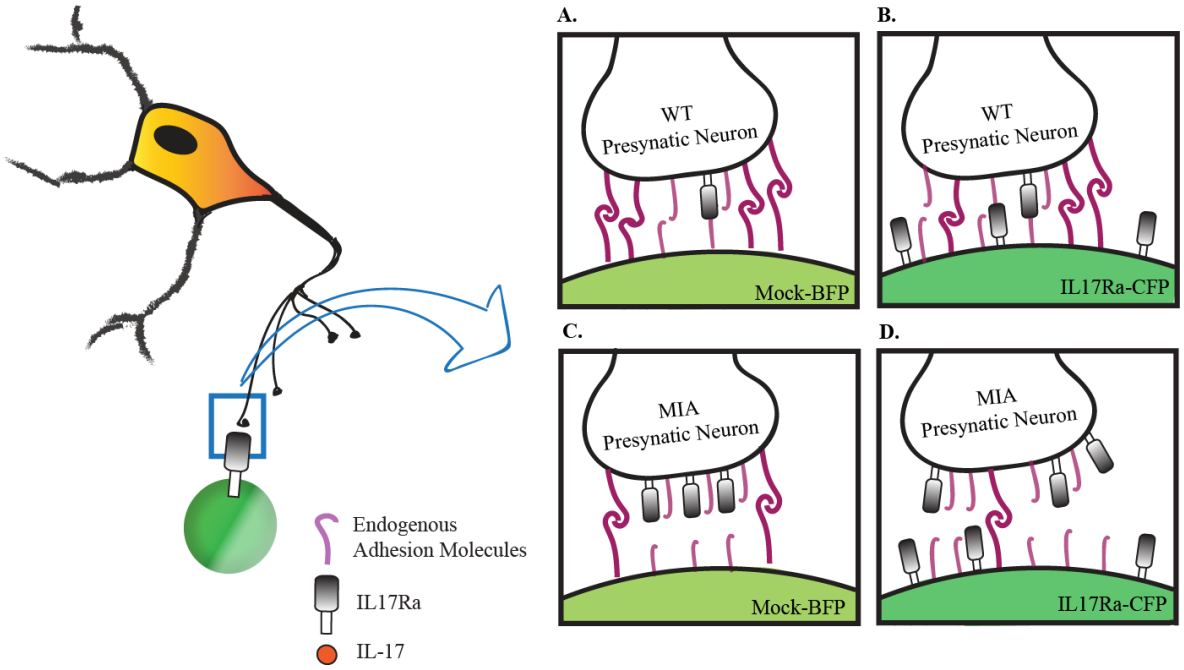


Figure 23: A proposed model for the role of IL17Ra during synapse formation. (A) Stable synapse formation between wild type primary cortical neurons and non-neuronal cells requires the aid of endogenous cell adhesion molecules. (B) This stable cell-to-cell synapse formation is disrupted when IL17Ra is overexpressed in the non-neuronal cell. (C) In MIA neurons that presynaptically overexpress IL17Ra, synapse formation is even more inhibited. (D) This inhibition effect is further amplified when the non-neuronal cell overexpresses IL17Ra as well.

CHAPTER 5: GENERAL DISCUSSION

Potential role of IL17Ra in the pathogenesis of ASD

The IL-17 cytokine family plays an important role in the regulation of immune response. A recent study identified the cellular source of the cytokine IL-17 that is necessary and sufficient to induce ASD-like phenotypes in MIA offspring, and showed that the mRNA levels of the cognate receptor of IL-17, IL17Ra, in the offspring are elevated upon MIA (*33*). Further elucidation of the IL17Ra signaling pathway would allow better understanding of how the downstream mediators induce abnormal cortical and behavioral phenotypes. Here, our results reveal an interesting link between immune response and the role of IL17Ra in the brain, as IL17Ra may have differing roles in various neurodevelopmental processes of cell adhesion, neurite outgrowth, and synapse formation.

It is worthwhile to note that some studies have suggested that IL17Ra may have normal developmental functions, and it is the overexpression and thus the hyperactivity of IL17Ra that disrupts normal neurodevelopment (*33, 45*). On the same note, here we propose a mechanism by which IL17Ra is involved in normal neurodevelopment, and upon overexpression, can lead to disruption in various neurodevelopmental processes. More specifically, we hypothesize that IL17Ra acts as an inhibitory protein that disrupts cell adhesion, and therefore possibly disrupts critical processes that require adhesion such as neurite outgrowth and synapse formation. For example, IL17Ra may activate downstream pathways that disrupt cell adhesion of other endogenous cell adhesion molecules that facilitate adhesion between cells (Fig. 24A). IL17Ra may also activate proteases that downregulate the expression of other cell adhesion molecules (Fig. 24B). Finally, our data most strongly suggest that IL17Ra has other binding partners that upon interaction destabilize cell adhesion (Fig. 24C). A growing body of evidence supports the

notion that IL17Ra signaling is critically involved in inducing ASD, and further studies are required to elucidate the exact mechanisms of this pathway.

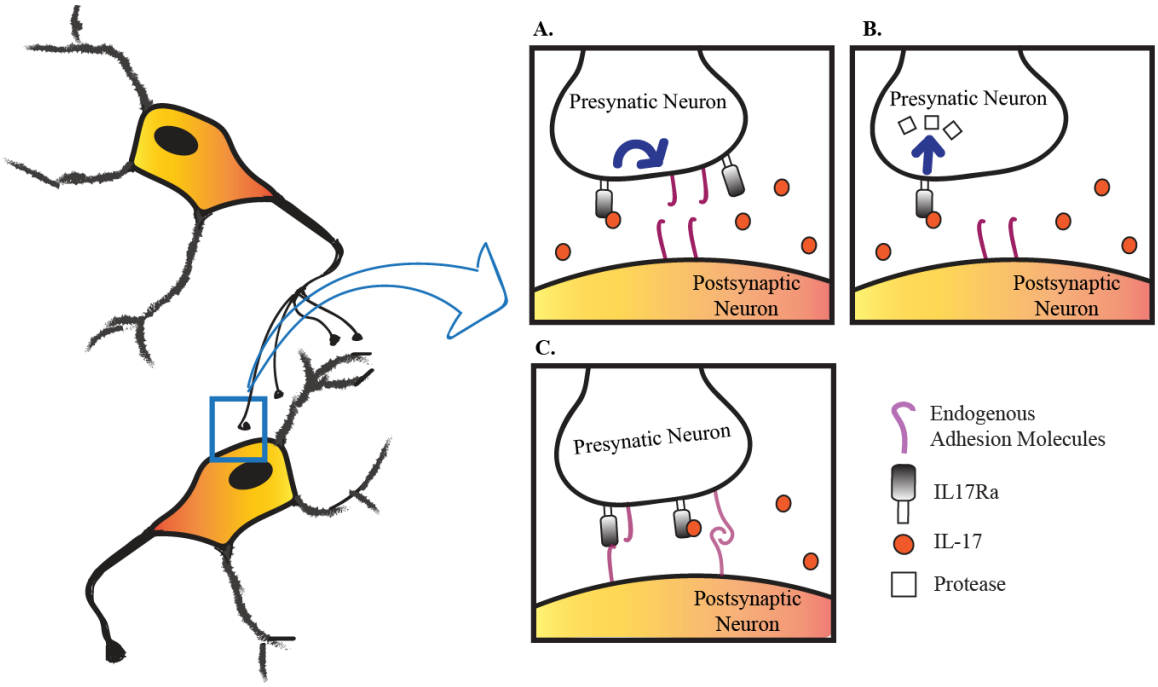


Figure 24: The potential role of IL17Ra in during cortical development. (A) IL17Ra activates downstream pathways to disrupt the interaction between other endogenous cell adhesion molecules. (B) IL17Ra activates proteases that down-regulate the expression of other endogenous cell adhesion molecules. (C) IL17Ra has other binding partners that upon binding, destabilizes adhesion. However, when its ligand IL-17, for which it has higher affinity for, binds, IL17Ra no longer binds to its binding partner and cell adhesion is not disrupted.

Consistent with our results, many other studies also support the notion that cytokine-induced disruptions of neurodevelopmental sequences underlie the pathogenesis of ASD. For example, studies attribute one of the contributing factors of ASD to the dysregulation of the cell cycle, as shown by increased proliferation of radial glial cells (*51*) or decreased number of precursor cells, due to cytokine dysregulation and abnormal microglial activity (*52*). Additionally, others attribute the disruption of cell migration, and therefore disorganized laminar positioning, to the cause of ASD (*53*). On a similar note, abnormal cortical phenotypes observed in MIA-induced ASD-like offspring showed corresponding patterns of disorganized cortical

patches seen in postmortem analyses of human ASD brains (*33, 44*). Finally, studies also show that the disruption of cell adhesion between synapses, and therefore the failure to initialize, specify, maintain, or regulate synapse formation, may underlie the pathogenesis of ASD. Consistent with this notion, recent studies indicate that various synaptic cell adhesion molecules may be candidate genes for various cognitive consequences in ASD (*41, 54-56*). Most importantly, another receptor of the cytokine family, IL1RcP, is a well-characterized cell adhesion molecule that organizes neuronal synaptogenesis (*41*). This suggests the possibility that cytokine signals converge on similar proteins to regulate processes of development, such as synaptogenesis.

Therapeutic and preventative implications

Currently, therapeutic intervention primarily focuses on behavioral therapies to control the behaviors of individuals affected with ASD. Moreover, some preventative measures that already exist include getting vaccinated, improving personal hygiene, and using contraceptives to prevent illnesses during gestation (*57*). Preventative and therapeutic intervention, as well as accurate diagnostic measures of ASD are all critically lacking.

Recent research, however, has strongly established the role of MIA as an underlying risk factor of ASD. Although more studies are required, these results that link maternal immune response and ASD has significant public health implications. Many studies have revealed the detrimental effects of the elevated cytokine levels, as well as have succeeded in manipulating cytokine levels to prevent ASD-like behaviors after MIA induction (*18, 33, 58*). For example, blocking IL-17 activity in the mothers via knockout models or treating mothers with antibodies resulted in successful prevention of ASD-like phenotypes in the MIA model (*33*). Identification of Th17 cells and IL-17 as key regulators not only highlights a potential therapeutic target for

pharmacological intervention, but also at the potential biological marker for diagnosing ASD and early intervention. However, more research is needed since cytokines are extremely versatile, and thus may lead to unexpected consequences elsewhere (*11*). Further elucidation of the Th17/ IL-17 signaling pathway may eventually yield successful diagnostic measures and preventative interventions, as targeting of Th17 cells in conjunction with regulating IL17Ra expression in susceptible pregnant mothers may allow us to reduce the likelihood of bearing children with inflammation-induced ASD phenotypes.

MATERIALS AND METHODS

Animals

All experiments were performed according to the Guide for the Care and Use of Laboratory Animals and were approved by the Committee and Animal Care at the Massachusetts Institute of Technology. C57BL/6 mice were obtained from Taconic (USA).

Maternal Immune Activation

Mice were mated overnight and females were checked daily for the presence of seminal plugs, noted as embryonic day 0.5 (E0.5). On E12.5, pregnant female mice were weighed and injected with a single dose (20mg/kg; i.p.) of poly(I:C) (Sigma Aldrich) or saline vehicle.

Cell Preparation

Human embryonic kidney (HEK) cell line 293FT (Life Technologies) was cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone), 50mM HEPES, 50U penicillin-streptomycin (Invitrogen), and 2 mM glutamine. 293FT cells were seeded into 6-well plates (Corning) 24 h prior to transfection at a density of $1x10^6$ cells/well. In preparation of cell aggregation assay and primary cultured neuron artificial synapse assay, cells were transfected with 1500ng plasmids/well using Lipofectamine 2000 (Life Technologies) following the manufacturer's recommended protocol.

Coverslips were incubated in 100% EtOH for 30 min at 37°C. Sterilized coverslips were then coated overnight with PDL (Sigma) at 37°C. Wild-type or MIA E12.5 embryos at each implantation site were dissected in ice-cold dissection media (DM) (HBSS (Invitrogen) containing 1% (v/v) HEPES, and 50U penicillin-streptomycin (Invitrogen)). Dissected cerebral cortical tissue were then minced and enzymatically dissociated in DM containing 2% Trypsin (Invitrogen) for 12 min at 37°C with intermittent mixing. Digested tissues were washed with DM and centrifuged 1000rpm for 1 min at 4°C three times. After washing coated coverslips with PBS, cells ($5x10^4$ cells/mL) were seeded into each of the 24-well plates (Corning) and cultured for 4 days in neurobasal media (Invitrogen) supplemented with 2mM glutamine (Sigma) and 1mM B-27 (In.vitrogen). In preparation of primary cultured morphology and synapse assays, IL-6 (Ebioscience) and IL-17a (Biolgened) were measured according to the manufacturer's protocol.

Cell Aggregation Assay

Transfected 293FT cells were trypsinized and centrifuged at 2000rpm for 1 min. The pellet was resuspended in aggregation harvest buffer (DMEM supplemented with 50mM HEPES and 10% FBS with or without 10mM $CaCl_2$). Different transfected 293FT cells were combined into 1500μL microcentrifuge tubes, treated with PBS, IL-17a, or IL-6, and placed on shaker for 24 h. Samples were taken at 0, 2, 4, 24 h upon resuspension and imaged using a confocal microscope (LSM710; Carl Zeiss) with a 10X objective lens; all image settings were kept constant.

Images were then analyzed using the software program ImageJ. In the control group, the areas of all single cells and cell aggregates at each imaging time point were measured (Fig. 9A). The averaged area of the single cells at 0 h was defined as baseline. Baseline values were eliminated from 2, 4, and 24 h data to normalize across the repeated trials, and then percent difference of

cell area from baseline was calculated to determine the normalized degree of cell aggregation. This analysis procedure was repeated for the IL17Ra overexpressing experimental group.

Primary Cultured Neuron Morphology Assay

4 days in vitro (d.i.v) after WT primary neuronal cell preparation, IL-17Ra (12 wells) or LacZ (12 wells) was knocked down using CRISPR/Cas9 genome editing as described elsewhere (59). Each IL-17Ra or LacZ knockdown group was treated with IL-17a (4 wells; 50µg/mL), IL-6 (4 wells; 50µg/mL), or PBS (4 wells) at d.i.v. 5. Fresh neurobasal media was added every 4 days. Each of the treatment groups was fixed at d.i.v. 6, 9, 12, and 15 with 4% paraformaldehyde in PBS for 10 min at 4^{o}C in preparation for immunocytochemistry.

Cells were permeabilized with blocking solution containing 0.4% Triton X-100, 2% goat serum, and 1% BSA in PBS for 1 h at room temperature, and then incubated with anti-beta actin (ab8229, Abcam), anti-synapsin I (ab8, Abcam) and anti-GFP (ab13970, Abcam) antibodies diluted 1:1000 overnight at 4 ^{o}C. The following day, cells were incubated with fluorescently conjugated secondary antibodies (Invitrogen, USA) diluted 1:250 for 1 h at room temperature, and mounted in vectashield mounting medium with DAPI (Vector laboratories). Images of stained cells were acquired using a confocal microscope (LSM710; Carl Zeiss) with a 20X objective lens; all image settings were kept constant.

Images were then analyzed using ImageJ. For the neurite outgrowth assay, the lengths (µm) of neurites, defined as processes between two somas, were measured and averaged for each experimental group (Fig. 13B). For the synapse puncta count assay, the number of puncta (represented by synapsin I expression) within 50µm of the neurite was measured and averaged for each experimental group (Fig. 17B).

Primary Cultured Neuron Artificial Synapse Assay

At d.i.v. 4 after WT or MIA neuronal cell preparation, fresh neurobasal media was added. At d.i.v. 9, 293FT cells were transfected with Lipofectamine 2000 and 1500ng of plasmids/well following the manufacturer's recommended protocol. At d.i.v. 10, transfected 293FT cells were trypsinized and centrifuged at 2000rpm. The 293FT cells were resuspended in fresh neurobasal media and 2x10^{3} 293FT cells were added to each neuronal well plate. Fresh neurobasal media were added every 4 days. At d.i.v. 13, cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4^{o}C in preparation for immunocytochemistry.

Fixed samples were then permeabilized with blocking solution containing 0.4% Triton X-100, 2% goat serum, and 1% BSA in PBS for 1 h at room temperature, and then incubated with anti-synapsin I (ab8, Abcam) diluted 1:1000 overnight at 4 ^{o}C. The following day, cells were incubated with fluorescently conjugated secondary antibodies (Invitrogen, USA) diluted 1:250 for 1 h at room temperature, and mounted in vectashield mounting medium (Vector laboratories). Images of stained cells were acquired using a confocal microscope (LSM710; Carl Zeiss) with a 40X objective lens; all image settings were kept constant.

Images were then analyzed using ImageJ. Co-localization of BFP (control) or CFP (IL17Ra) and synapsin I was used as a measure of synapse formation (Fig. 20B). Measurements of synapse formation was averaged and compared across different experimental groups.

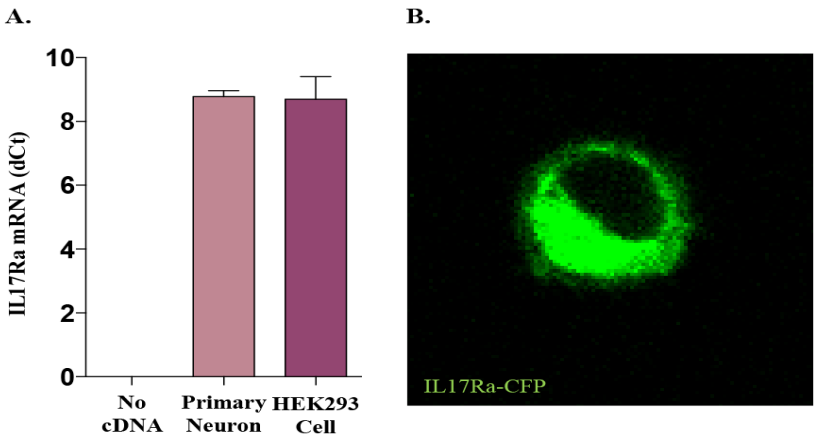
Statistical analysis

Results from cell aggregation assay and primary neuron culture assays were analyzed using unpaired, paired t-tests, and one-way ANOVAs. When statistical comparisons were made between two groups, an unpaired t-test was used. Probabilities of $P<0.05$ were considered significant.

SUPPLEMENTAL SECTION

Confirmation of IL17Ra expression levels in HEK293 cells

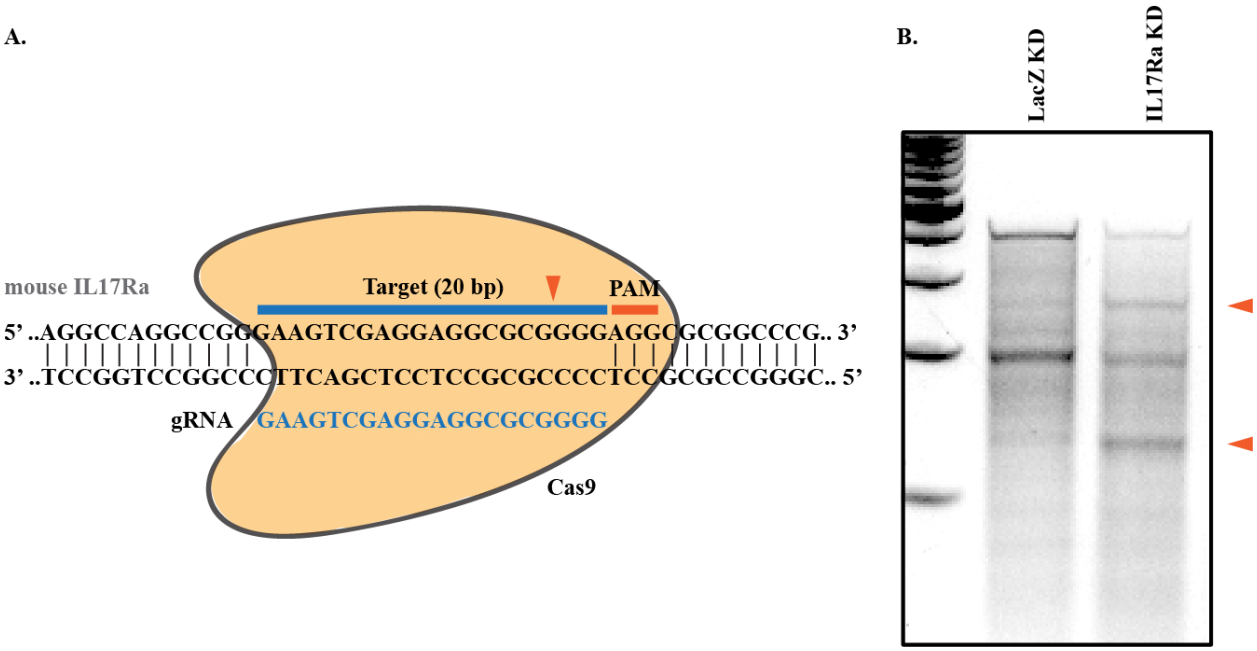
The endogenous IL17Ra levels in HEK293 cells were determined via real-time PCR (RT-PCR). Wild type primary cortical neurons and HEK293 cells endogenously express the same level of IL17Ra (Fig. S1). In HEK293 cells, IL17Ra expression is primarily localized to the membrane (Fig. S2).



Supplemental Figure 1: HEK293 cells and primary cortical neurons endogenously express similar levels of IL17Ra. (A) Primary cortical neurons (pink) and HEK293 cells (purple) express same endogenous levels of IL17Ra. Real-time PCR was done to determine levels of IL17Ra expression. All bars indicate mean $\pm$ s.e.m. (B) IL17Ra expression is localized to the cell membrane in IL17Ra transfected HEK293 cells.

Confirmation of IL17Ra KD in primary cortical neurons

20-nucleotide guide RNA (gRNA) was designed to target *il17ra* using the CRISPR/Cas9 single guide strategy targeting technique (Fig. S2A). Because a reliable IL17Ra antibody is yet to be made, CRISPR/Cas9 genome editing of IL17Ra was confirmed via a SURVEYOR nuclease assay. The SURVEYOR gel shows successful modification at the gRNA loci in wild type primary cortical neurons upon genome editing (Fig. S2B).



Supplemental Figure 2: CRISPR/Cas9 single guide strategy targeting *il17Ra.* A. The expected target sequence of Cas9 and its guide RNA (gRNA). B. SURVEYOR nuclease assay confirmation of IL17Ra genome editing in wild type primary cortical neurons, as indicated by gRNA loci (orange arrow).

Statistics:

Figure 11: Cell Aggregation Assay – PBS Treatment

Time point	Statistical Analysis
2 HR	Unpaired t-test; $p<0.01$, $t=3.148$, $df=362$
4 HR	Unpaired t-test; $p<0.001$, $t=3.681$, $df=388$
24 HR	Unpaired t-test; $p<0.0001$, $t=4.305$, $df=171$

Figure 12: Cell Aggregation Assay – IL-17 Treatment

Time point	Statistical Analysis
2 HR	Unpaired t-test; $p<0.05$, $t=2.022$, $df=256$
4 HR	Unpaired t-test; $p<0.0001$, $t=4.632$, $df=400$
24 HR	Unpaired t-test; p-0.121, $t=1.561$, $df=124$

Figure 13: Cell Aggregation Assay – Combined
One-way ANOVA; $p<0.001$, $F(3, 295)=6.795$

Figure 16: Neurite Outgrowth Assay – PBS vs. IL-17 Treatment

Group	Statistical Analysis
LacZ KD	Paired t-test; $p<0.0001$, $t=6.529$, $df=34$
IL17Ra KD	Paired t-test; $p<0.0001$, $t=5.694$, $df=63$

Figure 17: Neurite Outgrowth Assay – LacZ KD vs. IL17Ra KD

Treatment Type	Statistical Analysis
PBS	Paired t-test; $p<0.01$, $t=3.418$, $df=34$
IL-17	Paired t-test; $p<0.0001$, $t=7.317$, $df=116$

Figure 20: Synapse Puncta Assay – PBS vs. IL-17 Treatment

Genetic Group	Statistical Analysis
LacZ KD	Paired t-test; $p=0.7311$, $t=0.3479$, $df=23$
IL17Ra KD	Paired t-test; $p<0.0001$, $t=5.222$, $df=43$

Figure 21: Synapse Puncta Assay – LacZ KD vs. IL17Ra KD

Treatment Type	Statistical Analysis
PBS	Paired t-test; $p=0.0857$, $t=1.796$, $df=23$
IL-17	Paired t-test; $p<0.01$, $t=3.06$, $df=43$

Figure 23: Synapse Puncta Assay – IL17Ra overexpressing vs. Mock (Control)

Animal Type	Statistical Analysis
MIA	Unpaired t-test; $p<0.0001$, $t=5.471$, $df=66$
WT	Unpaired t-test; p-=0.6325, $t=0.4828$, $df=32$

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